THE ANTIBACTERIAL AND CHEMOATTRACTANT ACTIVITIES OF MURINE β-DEFENSINS

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2003

Declaration

I declare that this thesis has been composed by me and that all of the work is my own, unless otherwise stated.

Mark J. Rolfe

September, 2002


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I DON'T CARE ABOUT THINGS YOU CAN'T DO.
I KNOW. BUT IT MAKES A BETTER DEMONSTRATION THIS WAY.
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I WANT MY LIFE TO HAVE MEANING!
YOU CAME TO THE WRONG PLACE.
CAN'T DO THAT.

Acknowledgements

As many of you will know I don't normally do 'sincere', but in honour of this special occasion I shall try; but, to be honest it's unlikely that I will see any of you again, so if I fail I don't care.

Sir Winston Churchill once said "We have before us an ordeal of the most grievous kind; we have before us many long months of suffering and struggle". Of course, he was then referring to the war with Germany, but had he been born over a century later, in a different part of the country, led a totally different light and been called Julia, Fiona or Gillian, he may very well have been referring to my arrival in the lab in 1998. The fact that I am sat here today is testament to the skill, and to be frank spiritual strength of my colleagues.

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I would like to dedicate this thesis to the memory of my Granddad.

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List of Abbreviations

APC	Antigen Presenting Cell
ASL	Airway Surface Liquid
ATP	Adenosine Triphosphate
BAC	Bacterial Artificial Chromosome
BALF	Bronchoalveolar Fluid
CAP	Catioinc Antimicrobial Peptide
CCR	CC Chemokine Receptor
cDNA	Complementary Deoxyribonucleic Acid
cen	Centromere
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator Gene
CFU	Colony Forming Units
CXCR	CXC Chemokine Receptor
d6iDC	day-6 Immature Dendritic Cell
d7iDC	day-7 Immature Dendritic Cell
Da	Daltons
DCs	Dendritic Cells
DEFB	Human β-Defensin Gene
DEFB	Human β-Defensin Peptide
Defb	Murine β-Defensin peptide
Defb	Murine β-Defensin peptide
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ECP	Eosinophil Cationic Peptide
EDN	Eosinophil Derived Neurotoxin
EDTA	Ethylenediaminetetraacetic Acid
ENaC	Amiloride-Sensitive Epithelial Sodium Channel
FACS	Fluorescence-Associated Cell Sorting
FCS	Foetal Calf Serum
fMLP	formyl-Methionine-Leucine-Proline

FOV	Field of View
Gal	Gallinacin
Gal	Gallinacin Gene
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
HBD	Human β-Defensin Peptide
HBD	Human β-Defensin Peptide Gene
HBE	Human Bronchial Epithelial Cell Line
HBS	HEPES Buffered Saline
HBSS	Hanks' Buffered Salt Solution
HD	Human Defensin
HD	Human Defensin Gene
HEPES	N-2-Hydroxyethylpiperazine-N'-2ethanesulphonic Acid
HGMP	Human Genome mapping Project
HNP	Human Neutrophil Peptide (Protein)
iDC	Immature Dendritic Cell
IFN	Interferon
IL	Interleukin
LAP	Lingual Antimicrobial Peptide Gene
BNBD	Bovine Neutrophil β-Defensin Gene
LAP	Lingual Antimicrobial Peptide
BNBD	Bovine Neutrophil β-Defensin
$LD_{50 \text{ or } 90}$	Lethal Dose 50 or 90 (Dose required to kill 50% or 90% of test Animals)
LMP	Low melting Point
LPS	Lipopolysaccharide
LTA	Liopteichoic Acid
MACS	Magnetic-Associated Cell Sorting
mDC	Mature Dendritic Cells
MHC	Major Histocompatibility Complex
MI	Migratory Index
MIC	Minimum Inhibitory Concentration
MIP	Macrophage Inflammatory Protein
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MS	Mass Spectroscopy
NaCl	Sodium Chloride

NF-κB	Nuclear Factor κB
NK	Natural Killer
nm	nanometre
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PAMPs	Pathogen Associated Molecular patterns
PB	Phosphate Buffer
pBD	Porcine β-Defensin Gene
pBD	Porcine β-Defensin Peptide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNK	Polynucleotide Kinase
PR	Proline-Rich
PRR	Pattern recognition Receptors
RBC	Red Blood Cell
RNA	Ribonucleic Acid
rNP	Rabbit Neutrophil Peptide
rNP	Rabbit Neutrophil Peptide Gene
rRNA	Ribosomal Ribonucleic Acid
RTD	Rhesus θ-Defensin Gene
RTD	Rhesus θ-Defensin Peptide
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium Dodecyl Sulphate
Ta	Annealing Temperature
TAP	Tracheal Antimicrobial Peptide
TAP	Tracheal Antimicrobial Peptide Gene
TE	10 mM Tris.hydrochloric Acid/1 M EDTA Solution
tel	Telomere
TFA	Trifluoroacetic Acid
Tlr	Toll-like Receptor
Tm	Melting Temperature
TNFα	Tumour Necrosis factor
μCi	Micro-Curie

Abstract

Antimicrobial peptides form an important aspect of the innate immune response of mammals. They function to kill invading microorganisms and in many cases activate other aspects of the innate and adaptive immune systems. One of the largest families of antimicrobial peptides is the defensins. In vertebrates, there are three subfamilies of defensins, termed α, β and θ based on the connectivity of the six conserved cysteines. The β-defensins, which are produced mainly by epithelial tissues and keratinocytes, came to wider interest when it was proposed that their loss of function might play a role in the pathogenesis of cystic fibrosis (CF) lung disease. Previous work has suggested that the airway surface liquid (ASL) of primary cultures of human airway epithelial cells possess salt-sensitive antibacterial activity and that this is impaired in CF individuals by elevated levels of sodium chloride. Further work has also suggested that the β-defensins secreted by the airway epithelia might comprise an important component of this salt-sensitive defence system.

The aim of this project was 1) to characterise the salt-sensitive antibacterial activity of members of the human and murine β-defensin subfamily, 2) to analyse their activity as chemoattractants, 3) to establish a cell culture-based system for the production of β-defensins to allow for greater analysis of their range of activities and 4) to verify the validity of novel human β-defensins identified by bioinformatics techniques.

In this thesis describes the characterisation of the salt-sensitive activities of synthetic human β-defensin 2 (DEFB2), mouse β-defensin2 (Defb2), and a novel β-defensin related 1, Defr1, which lacks the first of the canonical six cysteines are described against a range of CF-related pathogens. This work has concluded that a) DEFB2, Defb2 and Defr1 display, to varying degrees, salt-sensitive antibacterial activity. b) The differences observed between the activities of the peptides may represent the evolution of species-specific profiles of antibacterial activity for specific defensins. c) That the loss of in Defr1 of the first canonical cysteine does not result in loss of

antibacterial activity and, most interestingly, Defr1 also demonstrates activity against *B. cenocepacia* – a pathogen normally resistant to the activity of antimicrobials.

Data presented in this thesis also suggests that synthetic Defb2 and Defr1 show chemotactic activity to CD4+ T-lymphocytes and to immature dendritic cells. This work concludes that, like human β-defensins 1 and 2, the murine β-defensins, Defb2 and Defr1, can act as a bridge between the innate and adaptive immune systems.

In this thesis, expression pattern of five novel human β-defensins in a range of human tissues is also analysed. Evidence is presented that they are all expressed at high levels in the testis and that two of these genes are expressed at much lower levels a variety of other tissues. These data suggest that the β-defensins are an expanding, and potentially quite large, subfamily of genes, many of which are yet to be characterised in terms of their expression profile and the antimicrobial and chemotactic activities.

Chapter 1: Introduction

1.1 The Adaptive and Innate Immune Systems

Organisms defend themselves against pathogens and foreign particles by a multitude of immune mechanisms. The immune system can be divided into two aspects: the innate immune system and the adaptive immune system. Both systems serve to defend the host from microbial challenge.

Adaptive immunity, which responds specifically to a particular type of pathogen, is mediated by lymphocytes that express an array of receptors (T-cell receptors and immunoglobulins). Clonal elimination removes T-cells bearing a receptor that recognises host molecules, and thus establishes self-tolerance. Clonal expansion, the proliferation of a lymphocyte clone bearing an antibody or T-cell receptor specific for a particular antigen, permits the generation of large immune effector cells with reactive receptors that are targeted against the appropriate invading pathogen or foreign particle. Furthermore, following activation of the adaptive immune system some antigen-specific lymphocytes survive, becoming memory cells and thus the adaptive immune system possesses the ability to mount a more rapid response to an antigen upon second exposure.

The acquired immune system has arisen relatively recently in evolutionary terms and was built over the phylogenetically ancient innate immune system. The adaptive immune system developed before the separation of vertebrates and invertebrates and many multicellular organisms depend exclusively on it. It acts effectively without previous exposure to the pathogen and it confers protection against a broad range of pathogens. The invading

organisms are recognised by a series of specific pattern recognition receptors (PRRs), which recognise 'microbial patterns' – those features that are specific to microorganisms and distinguish them from multicellular organisms. Such features include the β-1, 3-glucan of fungi, the lipopolysaccharide (LPS) of Gram-negative bacteria and the phosphoglycan of parasites reviewed in (Kimbrell and Beutler, 2001).

Many PRRs have been identified in both vertebrates and invertebrates. They all function to signal the presence of an invading microorganism and to activate the innate immune system. One of the most powerful weapons of the plant innate immune system is the hypersensitive response, which is rapid and localised cell death at the site of infection; other aspects include the production of reactive oxygen species, cell wall strengthening and the production of antimicrobial proteins (reviewed in Cohn *et al.*, 2001). In insects such as *Drosophilia* the innate immune will include the activation of haemocytes, which migrate throughout the body and phagocytose or encapsulate invading microorganisms, and the activation of proteolytic cascades leading to clotting and melanisation. Furthermore, the fat body of insects (analogous to the liver) produces an array of antimicrobial peptides. In vertebrates however, the inflammatory response is the hallmark of an innate immune reaction. This is characterised by the production of cytokines and the activation of macrophages and granulocytes such as neutrophils; these cells invade the infected area and phagocytose microorganisms. Many cells, both circulating immune-related cells and those of epithelia also possess the ability to produce a cocktail of antimicrobial peptides, which can directly kill the invading organisms.

1.2 Antimicrobial Peptides

An important feature of the innate immune system and one common to plants, invertebrates and vertebrates is the production of antimicrobial peptides. Their widespread distribution throughout the plant and animal kingdom suggests that antimicrobial peptides have served a fundamental role in the evolution of multicellular organisms. Despite their ancient lineage, antimicrobial peptides have remained effective components of the immune system. Moreover, the range and variety of antimicrobial peptides produced by organisms is vast, and, as their main target is the microbial membrane, the evolution of resistance is thought to be unlikely, as it would involve the redesign of the microbes' membrane. Furthermore, the acknowledgement of these features of antimicrobial peptides has lead to considerable commercial and academic research into developing a new generation of therapeutic antibiotics based on these naturally occurring antimicrobials (Zasloff, 2002).

1.2.1 The Properties and Classification of Antimicrobial Peptides

Most antimicrobial peptides are encoded by single genes; however, the diversity in the primary structure is so great that the same peptide sequence is rarely isolated from two different species – even those that are closely related. Significant conservation in the preproregion of antimicrobial peptides is found and this suggests constraints exist of the sequences involved in cellular processing, folding and secretion of the peptides. However, single mutations in the sequence of the mature antimicrobial peptides can dramatically alter their activity. For example, the antibacterial activity of the α-defensin human neutrophil peptide 1 (HNP-1) has

significant antimicrobial activity against *Candida albicans* whereas the α-defensin peptide HNP-3 is almost inactive even though the two peptides differ by only one amino acid at the N-termini (Lehrer *et al.*, 1988). This is likely to increase the spectrum of activity of the peptides and may reflect the host's adaptation to unique microbial environments (Zasloff, 2002).

Although some examples of anionic antimicrobial peptides have been reported (Brogden *et al.*, 1996; Kalfa and Brogden, 1999), the vast majority of antimicrobial peptides are cationic and amphipathic in nature. They are defined by Hancock and Chapple (1999) as being 12 to 50 amino acids in length with a net positive charge of at least +2 due to an excess of the basic amino acids arginine, lysine and histidine over acidic amino acids. They also contain a high percentage of hydrophobic amino acid residues, reflecting the fact that these peptides interact directly with cell membranes. Despite their small size and common physical characteristics, cationic antimicrobial peptides appear to have arisen from multiple, independent sources, possibly through convergent evolution (Scott and Hancock, 2000; Zasloff, 2002). The diversity of cationic antimicrobial peptides is so great that it makes classification difficult. However, this can be done broadly on the basis of secondary structure. This gives four major classes - β-sheet structures that are stabilised by two or three disulfide bridges, α-helical peptides, extended helices (polyprohelices), and looped structures (Figure 1.1 and Table 1.1) (Falla *et al.*, 1996).

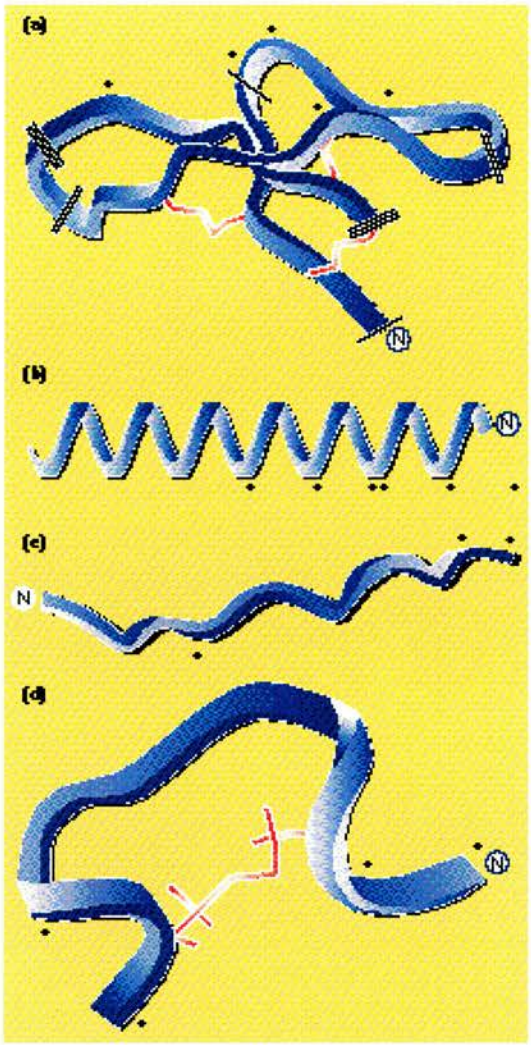

Figure 1.1: Structures of the four main classes of antimicrobial peptide. (a) β-stranded sheet of human β-defensin 1; (b) α-helical cecropin- melittin hybrid; (c) extended coil of indolicidin; (d) looped structure of bactenecin. The backbone of the structures is shown with positive charges (+), amino-termini (N), and in (a) and (d) the disulfide bridges are also indicated. In (a) there are three β-strands; their beginnings and ends are indicated by pairs of one, two or three lines on the backbone. Figure taken from Hancock (1997a).

Structural Class	Peptides	Details (Species; tissues)
Linear, α-Helical	Magainins	Amphibians; skins secretions
	Cecropins	Insects; larvae
	Pleurocidins	Flounder; skin secretions
Extended Coil	Indolicidin	Bovine neutrophils
	PR-39	Pig; intestine, neutrophils
Loop	Bactenicins	Mammals; leukocytes
	Polymyxins	Bacteria
β-Stranded Sheet	Defensins	Insects, Vertebrates; epithelial cells, leukocytes
	Tachyplesin	Crustacean (Horseshoe crab), haemocytes
	Protegrins	Pig, neutrophils

Table 1.1: Classification of Antimicrobial Peptides. Classification is based broadly on secondary structure. Four classes of peptide are discernable based on the secondary structure, relevant examples of each type are shown. Information in table adapted from Diamond (2001), Zasloff (2002) and Hancock (1997).

The most prominent class of antimicrobial peptide is the β-sheet structure, stabilised by two to four disulfide bridges and occasionally containing an α-helical region, this forms a relatively rigid structure with isolated patches of hydrophilic and hydrophobic amino acids residues. This structure is seen in peptides such as defensins (Selsted *et al.*, 1985). α-Helical peptides are also very common, however, many of these peptides such as the frog magainin (Zasloff, 1997) and the insect cecropin peptide (Steiner *et al.*, 1981) are linear, but when they enter a membrane they assume the amphipathic α-helical structure indicated in Figure 1.1. Less common are the extended coil peptides; these tend to have a predominance of more or more amino acids. For example, the pig antimicrobial peptide PR-39 is 39 residues long and contains 10 arginine residues, which are positively charged at neutral pH, and 19 proline residues. The final class of peptides have a looped structure formed by a single disulfide bond. An antimicrobial peptide found in frogs from *Rana esculenta* has an α-helical structure similar to the magainins, but with the addition of single a loop formed by a disulfide bridge at the

carboxy-terminus, and thus the peptide forms an 'R' shape (Simmaco *et al.*, 1994). Whereas, the bactenecin is a β-sheet stabilised by a single disulfide bond (Romeo *et al.*, 1998). Some antimicrobial peptides are generated by enzymatic digestion of much larger proteins, for example an antimicrobial peptide is generated from lactoferrin (Groenink *et al.*, 1999) and CAP18, which generates the antimicrobial cathelicidin LL37 (Larrick *et al.*, 1995).

1.3 The Defensin Family of Antimicrobial Peptides

One of the largest and most widely studied families of antimicrobial peptides is that of the defensins reviewed in (Lehrer and Ganz, 1999). Defensins were originally isolated from rabbit neutrophil granules as a result of experiments designed to characterise the oxygen-independent antimicrobial activities of phagocytes (Selsted *et al.*, 1984) and were also later isolated from the granules of human neutrophils (Ganz *et al.*, 1985). Subsequent studies isolated and characterised defensin molecules from plants (Broekaert *et al.*, 1995), insects (Bulet *et al.*, 1999), and many other vertebrate species (Lehrer and Ganz, 2002b). Defensin peptides fit the definition of cationic antimicrobial peptides stated above; they are approximately 37 amino acids in length, and have a net positive charge and they possess broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, some fungi and enveloped viruses.

1.3.1 Classification of Defensin Peptides

Small (3-6 kDa), cationic and cysteine-rich antimicrobial peptides have been termed defensins; the spacing of the cysteines and the arrangement of the disulfide bridges that stabilise the β-sheet structure are used to classify

defensins into different families and subfamilies (Table 1.2 and Figure 1.2) reviewed in (Ganz and Lehrer, 1994). Defensin peptides isolated from insects constitute a single family, whereas in vertebrates, defensins form at least three separate subfamilies: the originally isolated α-defensins (also termed classical defensins), the subsequently cloned β-defensins (Tang and Selsted, 1993) and the more recently identified θ-defensin subfamily, which to date contains only a single member (Tang *et al.*, 1999). Insect and vertebrate defensins all contain six cysteines, however, the spacing and arrangement of the cysteines differs reviewed in (Yang *et al.*, 2001). In insect defensins, the cysteines are linked C1 to C4, C2 to C5 and C3 to C6. In α-defensins, the cysteines are linked C1-C6, C2-C4 and C3-C5; the linkage of the cysteines in β-defensins is C1-C5, C2-C4 and C3-C6. In rhesus θ-defensin 1, the only θ-defensin isolated to date, the cysteines are linked in the pattern C1-C6, C2-C5 and C3-C4. Defensins have also been isolated form some plant species, however, these peptides contain eight cysteines, which form four disulfide bridges, the cysteines are linked C1-C8, C2-C5, C3-C6 and C4-C7 (reviewed in Yang *et al.*, 2001).

Classification		Origin	Intramolecular Disulfide Bridges	
Family	**Subfamily**		**Nº**	**Pattern**
Plant Defensins	-	Seeds, leaves	4	C1-C8, C2-C5, C3-C6, C4-C7
Insect Defensins	-	Fat body, larvae	3	C1-C4, C2-C5, C3-C6
Vertebrate Defensins	α	Avian and mammalian leukocytes Paneth cells	3	C1-C6 C2-C4, C3-C5
	β	Epithelial cells	3	C1-C5, C2-C4, C3-C6
	θ	Primate leukocytes	3	C1-C6, C2-C5, C3-C4

Table 1.2: Classification of β-defensins. Table details the classification of β-defensins; this is based upon the origin (organism and tissue) of the peptide as well as the number, spacing and connectivity of the conserved cysteine residues. Nº indicates the number of disulfide bridges present in the defensin molecule. Table taken from Yang et al. (2001).

1.3.2 The Structure of Defensins

Despite the differences in the structure of defensins detailed above, they all share a similar β-sheet structure, which is stabilise by the disulfide bonds. The defensin structure also demonstrates other features common to antimicrobial peptides: they have a net positive with arginine as the main basic amino acid, and an amphipathic structure with spatially isolated hydrophobic and charged regions (Ganz, 1999).

1.3.3.1 Mechanisms of Action

Defensins, and indeed antimicrobial peptides in general, have been described as membrane-active agents (Hancock and Lehrer, 1998), and they are all thought to operate by a similar mechanism. The initial, if not only, target of antimicrobial peptides is the cell membrane, which also forms the basis for their selectivity. Defensins, being positively charged, interact with the negatively charged components of microbial membranes. These components include LPS in Gram-negative bacteria, teichoic acid in Gram-positive bacteria, since these features are pathogen-associated molecular patterns (PAMPs) and not found in eukaryotic cells, defensins in general appear to be specific for prokaryotic membranes (Raj and Dentino, 2002). Furthermore, bacterial membranes are organised in such a fashion that the outer leaflet of the membrane is composed of a high proportion of anionic phospholipids (e.g. phosphatidylglycerol). This contrasts with the membrane of eukaryotic cells, in which the outer leaflet is composed primarily of Zwitterionic phospholipids that have no overall charge, although the inner leaflet is

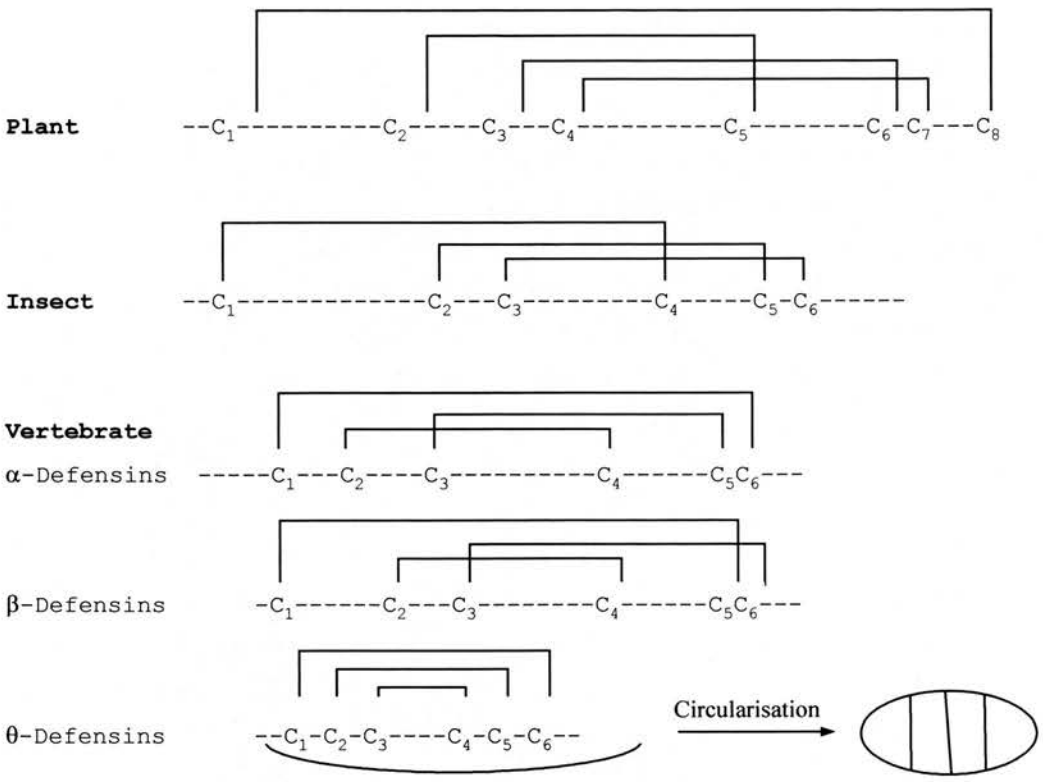

Figure 1.2: The Cysteine-Cysteine Pairing of the β-Defensin Families. Schematic diagram representing the connectivity of the different class of β-defensins peptides. The cysteines are numbered and represented by 'C, dashes (-) separating the cysteines represent the number of amino acid residues separating the cysteine residues in the peptide.

composed of negatively-charged lipids. This also means that antimicrobial peptides have a greater affinity for the negatively-charged bacterial membranes than the neutral membranes of eukaryotic cells. Moreover, the presence of cholesterol in mammalian membranes also reduces the potency of antimicrobial peptides, either by stabilisation of the membrane or by a more direct interference with the peptide-membrane interaction (Matsuzaki, 1999). Several different theories have been proposed to explain the antimicrobial mechanism of cationic peptides, and the precise mode of activity of defensins and other cationic peptides remains a subject of investigation.

The initial attraction between peptide and the target cell is established by the electrostatic interaction of the positively-charged peptide and the negatively-charged membrane. However, in Gram-negative bacteria, the peptides must first cross the outer membrane, and it has been suggested that this initial uptake occurs via self-promoted uptake (Hancock and Scott, 2000). This mechanism was first proposed to explain the uptake of polycationic antibiotics such as the aminoglycosides and polymyxins (Sawyer *et al.*, 1988). The peptides interact with the polyanionic surface of the outer membrane. As the peptides have an affinity for LPS that is at least three orders of magnitude greater than the native divalent cations, they competitively displace the Ca^{++} and Mg^{++} ions (Figure 1.3). Due to their bulk, the peptides cause disruption of the outer membrane, which 'cracks' and thus permits the uptake of the peptides into the periplasmic space (Hancock, 1997a). Interestingly, species belonging to the *Burkholderia cepacia* complex have been shown to be inherently resistant to the action of antimicrobial peptides and this is thought to be due to a nonreactive cell surface (Hancock, 1997a; Morrison *et al.*, 1998). In bacteria such as those of the *B. cepacia* complex the

LPS has low phosphate and high arabinosamine content, which precludes divalent cations and cationic antimicrobial peptide binding, and therefore self-promoted uptake (Hancock and Bell, 1988; Hancock, 1997b).

It is proposed that the peptides cross the periplasmic space, bind in parallel to the negatively charged outer leaflet of the membrane and cause membrane thinning of the leaflet. This causes the outer leaflet of the membrane to expand or bulge relative to the inner leaflet, which generates strain within the bilayer. The peptides then reorient, undergo a phase transition and insert into the microbial membrane with the hydrophobic face interacting with the hydrophobic core of the lipids and the charged surface of the peptide forming multimeric pores through which ions and other substances may pass (Figure 1.3). However, it is unclear exactly how the peptides insert, although it is thought to be driven by the large electrical potential across the bacterial membrane.

Two different models have been proposed to describe the structure of the peptide-membrane pore (reviewed in Ganz and Lehrer, 1999). In the barrel-stave model, the peptides oligomerise like the staves of a barrel and the complex behaves as a transmembrane protein. In the wormhole model, however, the peptides are embedded at the interface between the hydrophobic interior and the charged headgroups. This causes phospholipids to deform and bend from top to bottom and produces a pore lined with phospholipid headgroups and peptides (Ludtke *et al.*, 1996).

Several different hypotheses have also been proposed to explain how the peptides actually kill the target cells (Figure 1.3). One theory suggests that the killing event is the formation of the channels in the cytoplasmic

membrane, which is proposed to result in loss of cell viability (Falla *et al.*, 1996). An alternative theory, however, suggests that the antimicrobial peptides enter the cells through the disrupted membranes and interact with intracellular targets such as DNA (Hancock, 1997a).

Another theory has also been proposed to explain the antimicrobial mechanism of cationic peptides, which emphasises a different aspect of the peptide-membrane interaction (Huang, 2000). This theory proposes that the structural aspects of the interaction of peptide and membrane are of secondary importance, and the formation of wormhole pores is only a transitory event that transports peptides to the inner leaflet of the membrane, where they can interact with cellular targets or lead to further destabilisation of the membrane and its eventual disintegration (Figure 1.3). In this electrostatic-interaction theory (sometimes called the carpet model), the peptides aggregate into positively charged patches or carpets on the cell surface and neutralise the charge of the phospholipid headgroups over the area around the peptide aggregate. This neutralisation disrupts the integrity of the lipid bilayer and causes transient gaps to arise, which permits the passage of ions and larger molecules (Shai *et al*, 1999; Hoover *et al.*, 2000).

It is of course possible that all of these events are involved in the killing of the microbe, with the relative importance of the different events varying from cell to cell type, peptide to peptide and may also be dependent upon the peptide concentration.

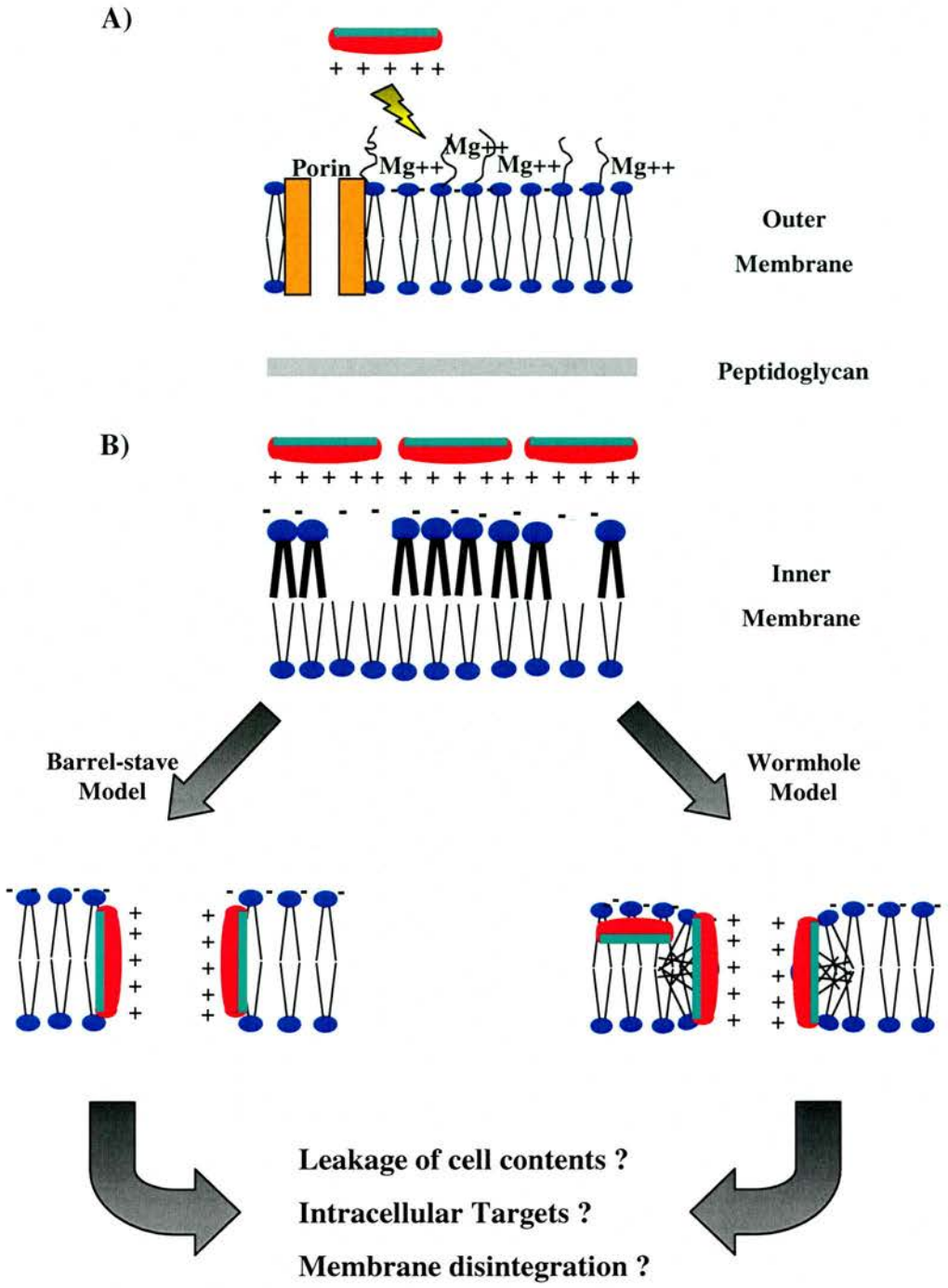

Figure 1.3: Schematic diagram showing the antimicrobial peptide's amphipathic structure and insertion into bacterial membrane.

A) Self-promoted uptake of peptides across the outer membrane must first occur in Gram-negative bacteria. The positively charged (red) peptide interacts with and displaces Mg^{++} ions, leading to the disruption of the outer membrane and peptide uptake. **B)** The peptides cross the periplasmic space and the charged face (red) of the peptide interacts with and carpets the outer leaflet of the membrane. This causes membrane thinning, which causes swelling of the outer leaflet of the membrane (in bold) and creates strain within the bilayer eventually leading to peptide insertion in the membrane. The peptides form pores in the bacterial membrane – either in the toroidal or barrel-stave fashion, which results in cell death either by leakage of cell contents or by interaction of the peptide with intracellular targets such as DNA. Figure is adapted from Hancock (1997) and Ganz and Lehrer (1999).

1.3.3.2 Mechanisms of Action - Defensins

The work described above was conducted using α-helical peptides such as magainins and defensin-like β-sheet peptides, however, this mode of action is proposed to be the mechanism by which most antibacterial peptides including defensins function (Raj and Dentino, 2002; Zasloff, 2002). One study demonstrates that treatment of *Micrococcus luteus* cells with the insect defensin, defensin A, results in inhibition of respiration and loss of cytoplasmic potassium and ATP, suggesting that defensins target the bacterial membrane, and they also form pores in the membrane (Cociancich *et al.*, 1993). Furthermore, it has also been demonstrated that α-defensins permeabilise the membranes of Gram-negative bacteria (Lehrer *et al.*, 1989). It has also been shown that they interact with anionic lipid vesicles, but not neutral vesicles through electrostatic and hydrophobic forces, which also fits the pore-formation model (Kagan *et al.*, 1990; Fujii *et al.*, 1993). A physical model of a human neutrophil α-defensin peptide (HNP3) has been constructed based on the x-ray structure of HNP3. This model suggested that 12 α-defensin monomers were arranged to form a membrane-spanning pore with an inner diameter of approximately 2 nm (Hill *et al.*, 1991).

The electrostatic interactions between defensins and the target cells suggest that specific membrane bound receptor interaction are unlikely, and this may explain why the occurrence of resistant bacterial strains is so rare. However, plant defensins have been shown to interact with specific binding sites on fungal cells (Thevissen *et al.*, 2000).

The three-dimensional-structure of several β-defensins have recently been elucidated and this has greatly increased the understanding of how this

subfamily of defensins may function (Hoover *et al.*, 2000; Hoover *et al.*, 2001; Bauer *et al.*, 2001). The human β-defensin 4 peptide has been shown to exist in both dimeric and octomeric (formed by four dimers) (Hoover *et al.*, 2000). Furthermore, the structural and electrostatic properties for the DEFB4 octomer, which is suggested to be the form bound to bacterial membranes, does not show any evidence for burial within the microbial membrane or the formation of membrane-spanning pores. The uniform distribution of positively-charged residues suggests that DEFB4 disrupts bacterial membranes by electrostatic interference as opposed to pore formation (Hoover *et al.*, 2000). Moreover, a uniform pattern of charge distribution is observed in the crystal structure of another human β-defensin, DEFB1 (Hoover *et al.*, 2001), and an analysis of the charge distribution of two murine β-defensins, Defb7 and Defb8, also failed to identify distinct hydrophobic and cationic regions that would be required for pore formation (Bauer *et al.*, 2001). Taken together these results suggest that the electrostatic-interference (or carpet) model, as opposed to the pore formation model, may be common to β-defensin peptides. There appears to be little evidence to support the mechanism proceeding by formation of membrane-embedded pores, and the full elucidation of the antimicrobial mechanism will require further experiments. However, a recent study suggested that DEFB103 forms functional ion channels in the membrane of *Xenopus* oocytes (Garcia *et al.*, 2001a)

1.3.4 The Mammalian Defensin Subfamilies

In humans, the defensin gene family is located on chromosome 8p23, and in the mouse at the syntenic region of mouse chromosome 8A4 (Huttner *et al.*, 1997; Liu *et al.*, 1998; Morrison *et al.*, 1999; Linzmeier *et al.*, 1999; Jia *et al.*, 2001). The different classes of mammalian defensins have evolved many differences however they are thought to share a common ancestry (Linzmeier *et al.*, 1999).

1.3.4.1 The α-Defensin Subfamily

α-Defensins were initially identified in granules of rabbit and human neutrophils and have subsequently been isolated from many other species including mice (Selsted *et al.*, 1984; Ganz *et al.*, 1985). α-Defensins are encoded by genes comprising three exons and are initially synthesised as prepropeptides (Lehrer *et al.*, 1993). These have an approximately 20 residue signal sequence for insertion into the endoplasmic reticulum, which is then cotranslationally removed, and a 40-45 amino acid propiece cleavage of which generates the functionally mature α-defensin peptide of approximately 30 amino acid residues. As discussed above the anionic propiece serves to neutralise the cationicity of the mature peptide and therefore may afford some protection from autocytotoxicity, and the α-defensins do not show antibacterial activity until cleavage of the propiece has occurred (Valore *et al.*, 1996).

Six human α-defensins have been isolated have been isolated to date (Lehrer and Ganz, 1999). Four of these, human neutrophil peptide (HNP) 1-4, are

expressed primarily by neutrophils, but expression has also been reported in B- and γδT-lymphocytes, monocytes/macrophages and natural killer cells (Ganz *et al.*, 1985; Wilde *et al.*, 1989; Agerberth *et al.*, 2000). In contrast, however, the other two α-defensin peptides, human defensin (HD) 5 and 6, are constitutively expressed by the secretory Paneth cells, which lie in the crypts of the gut epithelium. Expression of HD5 also has also been detected in the vagina and ectocervix and in the inflamed fallopian tube (Quayle *et al.*, 1998). Moreover, the expression of HD5 was found to peak during the secretory phase of the menstrual cycle, suggesting that expression may be regulated by hormones. Therefore, it appears that in humans the α-defensins contribute to systemic immunity (HNP1-4) and the defence of a limited number of epithelial surfaces (HD5 and 6). Human neutrophil defensins are among the most abundant neutrophil protein: HNP1-3 have been shown to make up 5-7% of total neutrophil protein and up to 50% of the protein content of the azurophilic granules of neutrophils. Indeed, their importance in neutrophil-mediated defence is suggested by specific granule deficiency. In this disease, a disorder of neutrophil development, the concentration of HNPs can be a low as 10% of the normal value and affected patients suffer from repeated infections by common bacteria (Johnston *et al.*, 1992). Furthermore, acquired defensin deficiency is occasionally observed in patients with chronic myelogenous leukaemia, however, its clinical consequences have not been explored (Borregaard *et al.*, 1993; reviewed in Ganz and Lehrer, 1994).

Mouse neutrophils do not express detectable levels of α-defensins (Eisenhauer and Lehrer, 1992), however mice do have an extended family of at least seventeen α-defensins (called cryptidins) that have been identified in the mouse gut and skin (Ouellette and Selsted, 1996; Shirafuji *et al.*, 1999). It

has recently been demonstrate that the matrix metalloproteinase matrilysin is required for activation of cryptidins in the gut via removal of the proregion (which is acidic and functions to neutralise the basic mature peptide). Peptides isolated from matrilysin knockout mice were demonstrated to have significantly impaired antimicrobial activity and orally administered bacteria survived to a greater extent in the matrilysin-deficient mice compared to wildtype mice. Furthermore, the LD_{50} of *Salmonella typhimurium* (the dose required to kill 50% of treated animals) for matrilysin knockout mice was 10% of that for wildtype mice; these data clearly suggesting that defensins play a significant role in intestinal defence (Wilson *et al.*, 1999).

Mature α-defensins have been shown to possess broad-spectrum salt-sensitive antibacterial activity against both Gram-positive and Gram-negative bacteria (Ganz *et al.*, 1985; Miyasaki *et al.*, 1990). Antimicrobial activity has also been demonstrated against fungi and some enveloped viruses (Daher *et al.*, 1986; Lehrer *et al.*, 1986; Lehrer *et al.*, 1988). The microbicidal activity has been shown to be inhibited by high salt-concentrations (Lehrer *et al.*, 1988; Lehrer *et al.*, 1993). However, killing by α-defensins may occur in phagoctyic vacuoles or at epithelial surfaces where salt concentrations may be low or where the concentration of defensin may be high enough to overcome the inhibition by salt (Ganz and Weiss, 1997).

1.3.4.2 Other Activities of α-Defensins

α-Defensins have also been shown to possess a range of other activities (Figure 1.4) (reviewed in van Wetering et *al.*, 1999 and in Yang *et al.*, 2001). Human neutrophil defensins have been shown to enhance phagocytosis by

macrophages (Ichinose *et al.*, 1996), and human, rabbit and guinea pig α-defensins have also been shown to induce histamine release and mast cell degranulation (Yamashita and Saito, 1989; Befus *et al.*, 1999). Human α-defensins can augment interleukin- (IL) 8 production and mast cell degranulation and IL-8 increase neutrophil influx into an inflamed area (van Wetering *et al.*, 1997a; van Wetering *et al.*, 1997b). Recent data has also shown that HNP1 and 2 are directly chemotactic for immature dendritic cells and naïve T-cells (Yang *et al.*, 2000a). Furthermore, human α-defensins can bind to complement factor C1q (Panyutich *et al.*, 1994) and either enhance or suppress activation of complement via the classical pathway, suggesting that α-defensins may also regulate the complement cascade. A further study has also been shown to enhance antigen-specific humoural immunity (Lillard., *et al.*, 1999). Intranasal delivery of human neutrophil peptides with ovalbumin enhanced ovalbumin-specific IgG antibodies production compared to animals treated with ovalbumin alone.

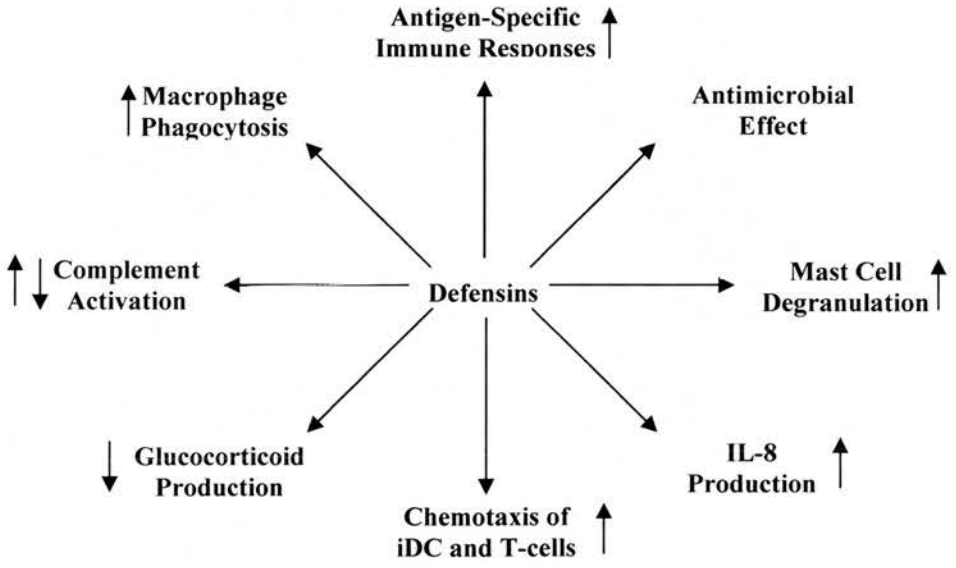

Figure 1.4: The Various activities of Mammalian Defensins. Spider diagram showing the diverse range of functions displayed by α-defensins; many of these functions have subsequently been shown for β-defensins. Where appropriate the arrows indicate an increase or decrease in activity. Figure taken form Yang *et al.*, 2001.

1.3.4.3 The θ-Defensin Subfamily

A recent study has identified a novel, circular, defensin subfamily expressed in *Rhesus* macaque leukocytes (Tang *et al.*, 1999). *Rhesus* theta-defensin 1 (RTD-1) is formed from two different α-defensin precursors. The precursor α-defensins are mutated in that they have acquired a stop codon between the

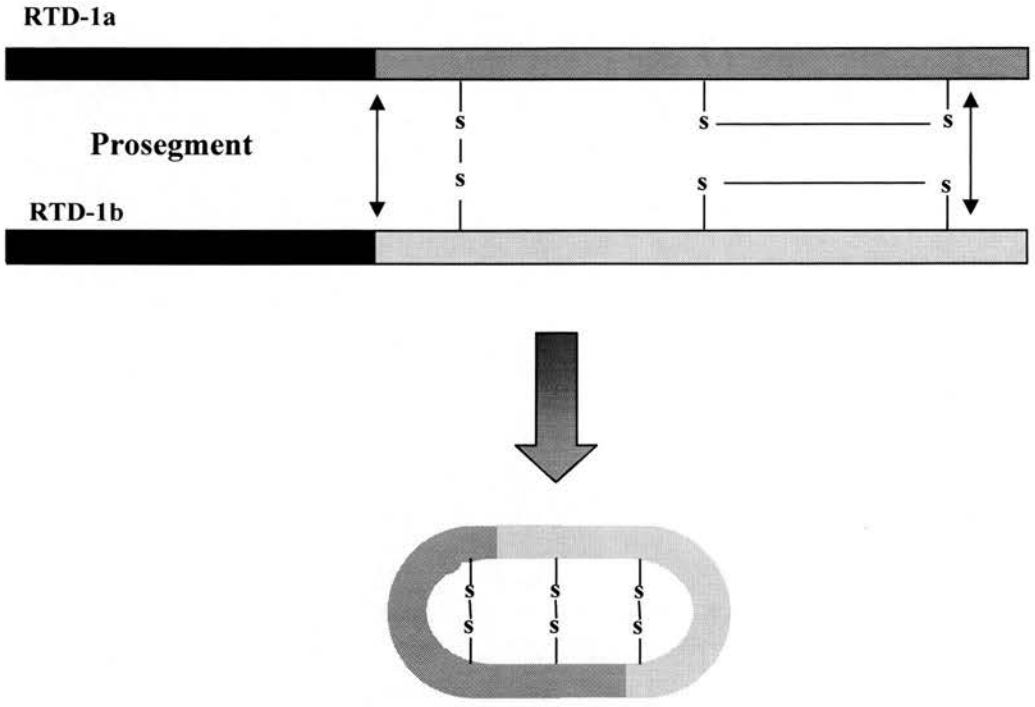

Figure 1.5: Posttranslational Processing of RTD1 Precursors. Small arrows indicate where RTD1a and RTD1b are spliced, generating two nonapeptides and then ligated to produce the circular mature RTD1. Homodimeric θ-defensins have also been reported Figure adapted from Tang *et al.*, 1999.

third and fourth cysteine. The precursors (called RTD1a and RTD1b) are translated; their ends are spliced and ligated together to produce a circular defensin (Figure 1.5). The processed peptide has 18 residues, including six cysteines that form intramolecular disulfide bonds. A recent study has also identified two homodimeric θ-defensin peptides (Tran *et al.*, 2002). The two new θ-defensins, termed RTD-2 and RTD-3, are formed from the

homodimeric splicing of RTD1b and RTD1a respectively. The cellular ratios of RTD-1, 2 and 3 were 29:1:2, indicating a clear preference for the heterodimeric RTD-1. However, the antibacterial activities of the three peptides were found to be broadly similar, except that RTD-2 demonstrated significantly lower activity against *Escherichia coli*.

Interestingly, the antibacterial activity of the cyclic RTD-1 was found to be salt-insensitive in sharp contrast to the salt-sensitive activity of the α-defensin peptides. It is also noteworthy that synthetic cyclic analogues of the rabbit neutrophil α-defensin, rNP-1, also retained antibacterial activity in the presence of salt-concentrations that inhibited the activity of the normal NP-1 peptide (Yu *et al.*, 2000). Moreover, the antimicrobial activity of the acyclic form of RTD-1 was inhibited by salt. The authors suggested that this was due to the presence of additional charges at the termini of the open chain analogue as opposed to overall structural differences (Tang *et al.*, 1999). This theory was supported when the three-dimensional structures of RTD-1 and the open-chain analogue of RTD-1 were elucidated. Furthermore, the structure of RTD-1 reveals that the peptide lacks a pronounced hydrophilic structure, and this may be an important factor in the salt-insensitive activity of RTD-1 (Trabi *et al.*, 2001).

1.3.4.4 The β-Defensin Subfamily

The first β-defensin to be identified was isolated some years after the initial isolation of α-defensins. It was cloned from cow trachea and termed tracheal antimicrobial peptide or TAP (Diamond *et al.*, 1991). β-Defensins were subsequently found in bovine granulocytes (bovine neutrophil β-defensins, BNBD1-13) (Selsted *et al.*, 1993), and in chicken leukocytes (gallinacin, Gal-1α

and Gal1-2) (Harwig *et al.*, 1994). They were distinguished from α-defensins by the spacing and connectivity of the six cysteines, which in BNBD12 and HBD4 are linked C1-C5, C2-C4 and C3-C6 (Table 1.2) (Tang and Selsted, 1993; Garcia *et al.*, 2001b). Further studies identified an inducible bovine β-defensin, expressed in the tongue, called lingual antimicrobial peptide (LAP) (Schonwetter *et al.*, 1995). Bovine and avian β-defensins show antimicrobial activity against both Gram-positive and Gram-negative bacteria *in vitro* at concentrations of 10-100 μg/ml (Ganz and Lehrer, 1995). A β-defensin has also been isolated from pigs (*pBD1*), which was found to be constitutively expressed at many epithelial surfaces including the respiratory tract, testis, kidney and digestive tract (Zhang *et al.*, 1998a; Zhang *et al.*, 1999). Functional analysis of pBD-1 showed that it possesses salt-sensitive antimicrobial activity at peptide concentrations of 20–100 μg/ml against bacteria such as *Salmonella typhimurium* and *Listeria monocytogenes* and that the protegrin peptide PR-39 can act synergistically with pBD-1 against *S. typhimurium* (Shi *et al.*, 1999). Two β-defensins have also been cloned from rats (termed *RBD1* and *RBD2*). *RBD-1* shows high similarity to the human β-defensin 1 and mouse β-defensin 1 and was abundantly expressed in the kidney, whereas *RBD2* shows greater homology to human β-defensin 4 and is prevalent in the lung (Jia *et al.*, 1999). Two β-defensins have also been cloned from sheep and also from goats (Huttner *et al.*, 1998; Zhao *et al.*, 1999). Taken together these data suggest that the β-defensins are a widely distributed and highly variable subfamily of antimicrobial peptides expressed at many different epithelial surfaces.

Until recently research into the activities of β-defensins lagged behind the study of α-defensins. However, several recent studies which implicated

dysfunction of human β-defensin 1 in the pathogenesis of cystic fibrosis (CF) have renewed interest in the study of this class of peptides (Smith *et al.*, 1996; Goldman *et al.*, 1997; Zabner *et al.*, 1998).

1.3.4.5 Human and Murine β-defensins

The β-defensin genes are located at the same genetic locus as the α-defensins: 8p22-23 in humans and the syntenic region 8A4 in mice (Figure 1.6A and C). The β-defensins are also produced as prepropeptides. However, unlike α-defensins the proregion does not appear to function to neutralise the mature peptide. This may be because β-defensins are not stored in granules prior to release, whereas the α-defensins are (Ganz and Lehrer, 1995). Moreover, the cleavage of the propiece does not appear to be necessary for antimicrobial activity. Indeed Valore *et al.* (1998) isolated from urine several different isoforms of DEFB1, ranging in length form 36 to 47 amino acids due to different amino-terminal truncations. The 36-residue peptide was the most abundant and also the most potent and least salt-sensitive isoforms, however the different isoforms demonstrated different levels of activity and antibacterial profiles. These data suggest that the proregion of DEFB1 may serve to alter the antibacterial spectra of DEFB1 and thus increase variation. This study also produced DEFB1 peptide using a baculovirus system and isolated different N-terminal isoforms from different cell lines, suggesting that posttranslational processing of DEFB1 may occur in a cell-specific manner.

1.3.4.6 The Human β-Defensins

In humans four β-defensins have been characterised in detail (Figure 1.6B), although the sequences of at least 28 more have been reported (Schutte *et al.*, 2002). The similarities and sequences of those β-defensins that have been characterised in detail are indicated in Table 1.4 and Figure 1.6A and B

The first human β-defensin to be identified was originally isolated from blood filtrate as a 36 amino acid residue peptide with a molecular mass of 3928.6 Da (Bensch *et al.*, 1995). The sequence of the cDNA showed homology to bovine TAP, BNBD1-12 and chicken gallinacins, including the six-cysteine motif, and thus was named human β-defensin 1 (*HBD1* or *DEFB1*).

Subsequently, *DEFB1* was found to be constitutively expressed high levels in the pancreas and kidney and at lower levels in the salivary gland, trachea, prostate, placenta thymus testis and small intestine (Table 1.3) (Zhao *et al.*, 1996). DEFB1 expression was located in the loops of Henlé and collecting ducts of the kidney (Schnapp *et al.*, 1998). These results suggest that it may play a role in the defence of epithelial surfaces and mucosa. The *DEFB1* gene is 7 kb in size, with a 6962 bp intron separating two exons, and is located 150 kb from the *HNP-1* gene (Liu *et al.*, 1997). DEFB1 has been shown to possess salt-sensitive antibacterial activity *in vitro* against *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* (Goldman *et al.*, 1997; Morrison *et al.*, 1998; Singh *et al.*, 1998), and its salt-sensitive activity has been implicated in the pathogenesis of CF lung disease (see section 1.3.5) (Goldman *et al.*, 1997).

The concentration of DEFB1 has been measured by Singh *et al.*, (1998) and detected at a concentration of 0.1 to 1 ng/ml in the broncheoalveolar lavage fluid. Studies have also suggested that DEFB1 possess cytotoxic activity towards mammalian cells, and that DEFB1 may also play a role in the eradicative processes at epithelia (Zucht *et al.*, 1998).

The second human β-defensin initially termed HBD2 or DEFB2 was isolated from psoriatic skin as a 41 residue peptide with 44% identity to DEFB1 (Table 1.4) (Harder *et al.*, 1997). However, as the peptide is the putative orthologue of mouse β-defensin 4 (*Defb4*) this gene was subsequently renamed *DEFB4* and that is the name used in this thesis. DEFB4 was found to exhibit highly effective killing of Gram-negative bacteria *E. coli* and *P. aeruginosa*, and also the yeast *Candida albicans*, but only a bacteriostatic effect against the Gram-positive *S. aureus*. Further studies have shown the antimicrobial activity of DEFB4 is salt-sensitive, although this sensitivity can be overcome by increasing the peptide concentration and moreover the activity is 10-fold greater than that of DEFB1 and is also synergistic with other aspects of the innate immune system such as lactoferrin and lysozyme (Harder *et al.*, 1997; Bals *et al.*, 1998b; Singh *et al.*, 1998). Characterisation of synthetic peptide based on the sequence of DEFB4 is reported in Chapter 3 of this thesis.

DEFB4 is expressed in the skin, lung and trachea and expression is induced upon exposure to bacteria and inflammatory stimuli and also in individuals with bacterial pneumonia (Table 1.3) (Harder *et al.*, 1997; Bals *et al.*, 1998b; Hiratsuka *et al.*, 1998). Expression of *DEFB4* has also been detected in several other tissues including urogenital tract, salivary gland and uterus (reviewed in Schroder and Harder, 1999). However, Singh *et al.* (1998) did not detect

DEFB4 in the broncheoalveolar lavage fluid of healthy individuals, but in patients with inflammatory lung disease or cystic fibrosis DEFB4 was present at a concentration of 1-10 ng/ml.

The *DEFB4* gene is located approximately 500 to 600 kb centromeric to *DEFB1* (Figure 1.6) and composed of two exons separated by a 1.6 kb intron (Liu *et al.*, 1998). Moreover, the flanking region of DEFB4 contains four NFκB sites, these are not found in the DEFB1 gene but are present in TAP and LAP, which like DEFB4 are upregulated by inflammatory stimuli (Russell *et al.*, 1996) and DEFB4 expression has been shown to be induced via by Toll-like receptor (Tlr2) 2 and CD14 (Becker *et al.*, 2000; Birchler *et al.*, 2001).

The third human β-defensin was identified by screening of genomic sequences using bioinformatics techniques and was initially termed *DEFB3*, but subsequently renamed *DEFB103* (Garcia *et al.*, 2001a). It was also independently purified from psoriatic lesional skin and subsequently cloned from keratinocytes (Harder *et al.*, 2001). The gene for *DEFB103* is composed of two exons, has a small intron of just 943 bp, and encodes a 67 amino acid peptide (Garcia *et al.*, 2001a). *DEFB103* was found to be expressed in the skin, tonsils, placenta, testis, trachea and oesophagus, and interestingly, expression was also found in neutrophils (Table 1.3). Expression is up regulated by interferon (IFN) –γ, but not by tumour necrosis factor (TNF-) α, or interleukin (IL) -1α or –6 (Garcia *et al.*, 2001a). DEFB103 has also been

TISSUE	*DEFB1*	*DEFB4*	*DEFB103*	*DEFB104*
Skin	+	$+++^{Ind}$	NR	NR
Muscle	NR	NR	++	NR
Lung	+	$+++^{Ind}$	+	+
Trachea	+	$+++^{Ind}$	++	$++^{Ind}$
Digestive	++	-	+	+++
Salivary	+	-	NR	NR
Kidney	+++	-	-	+
Liver	+	-	+	NR
Heart	NR	NR	++	NR
Testis	NR	NR	+++	+++

TISSUE	*Defb1*	*Defb2*	*Defb3*	*Defb4*	*Defb6*	*Defr1*
Skin	NR	NR	NR	-	NR	NR
Muscle	NR	NR	NR	NR	+++	NR
Lung	++	NR	$++^{Ind}$	-	-	-
Trachea	++	$++^{Ind}$	$++^{Ind}$	++	+	-
Digestive	NR	NR	+	+++	+	NR
Salivary	NR	NR	+	-	NR	NR
Kidney	+++	+++	NR	-	-	-
Liver	++	NR	$++^{Ind}$	-	-	-
Heart	+	+	NR	-	-	++
Testis	NR	NR	NR	NR	-	+++

Table 1.3: Pattern of expression of human and murine β-defensins. + = detectable expression, - = expression not detected, ^{Ind} = expression up-regulated following proinflammatory stimuli, NR = expression has not been reported. N.B. Levels of expression are only comparable for each gene and not between β-defensins.

		SIMILARITIY									
	Defensin	DEFB1	DEFB4	DEFB103	DEFB104	Defb1	Defb2	Defb3	Defb4	Defb6	Defr1
	DEFB1	-	47	36	33*	57	41	41	43	46	47
I	DEFB4	44	-	53	41*	42	36	57	59	53	60
D	DEFB103	33	45	-	41*	44	37	51	43	46	43
E	DEFB104	45*	41*	38*	-	38*	36*	46*	30	44	50*
N	Defb1	51	36	37	38*	-	50	39	39	48	43
T	Defb2	34	31	29	36*	41	-	38	45*	42*	50*
I	Defb3	36	49	46	39*	33	38	-	66	70	73
T	Defb4	34	48	39	28	30	45*	61	-	72	67
Y	Defb6	31	37	40	33	34	39*	59	61	-	68
	Defr1	40	52	38	32*	36	46*	72	63	60	-

Table 1.4: Percent similarities and percent identities of the different human and murine β-defensin prepropeptides. For similarity, between two peptides read vertically down and across. For identity, read across then vertically up. * Indicates that the peptide alignment does not include a significant length of the peptide sequences. Analyses were performed using the 'bestfit' function of GCG program at the HGMP website (www.hgmp.mrc.ac.uk).

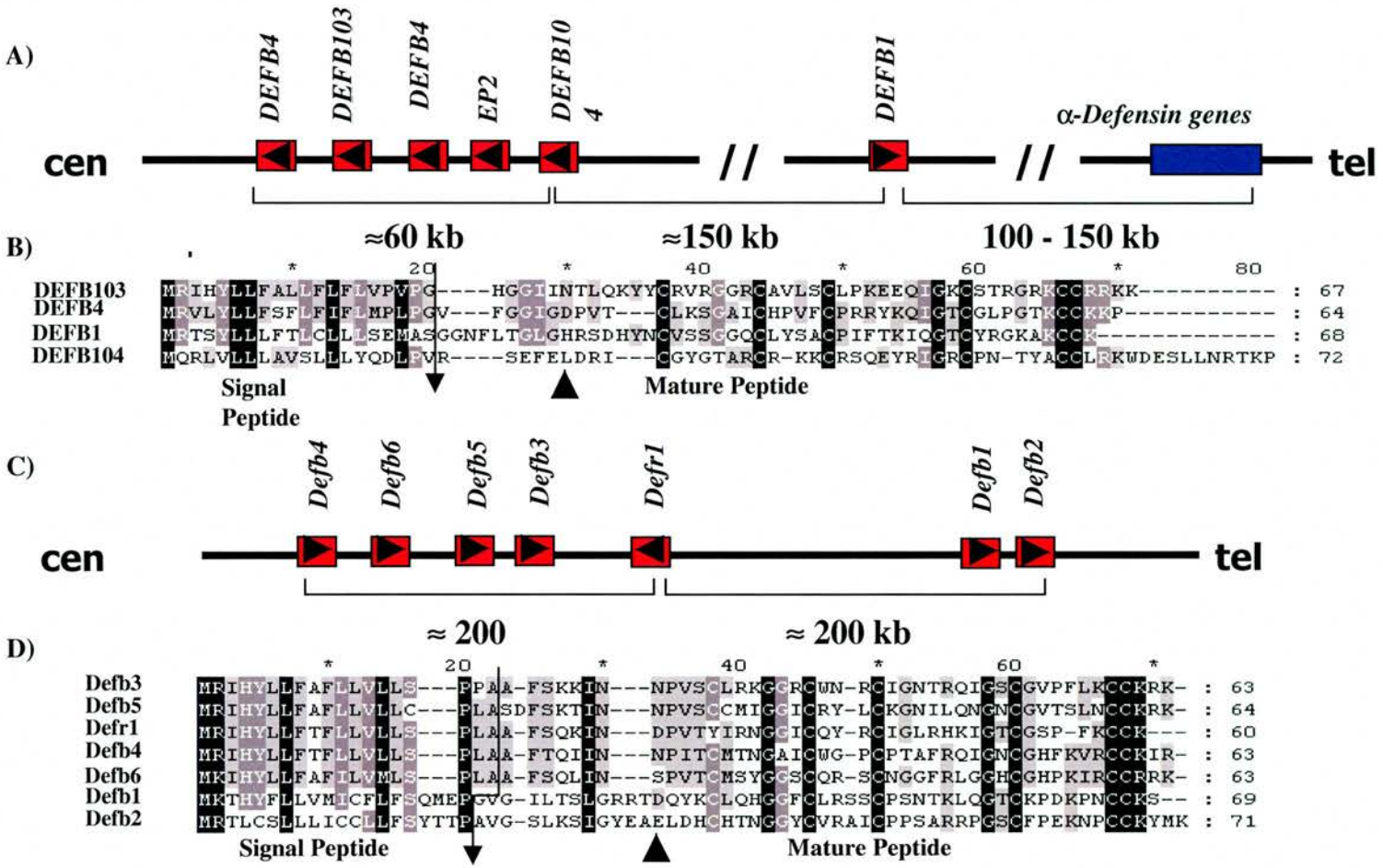

Figure 1.6: Sequences and Chromosomal loci of mouse and human β-defensins loci. A) and C) Schematic diagrams of the main human and mouse β-defensin clusters on syntenic regions of chromosome 8. Square brackets indicate the approximate genomic distances between the gene clusters, arrow heads represent orientation of the gene. B) and D) Comparison of the sequences of the human and mouse β-defensins. Arrows indicate the exon boundaries and the larger arrowheads indicate the predicted mature peptide sequence For simplicity, only the β-defensins reported in detail are shown here. The alignments were generated using Clustal via the HGMP website.

shown to possess salt-sensitive antimicrobial activity *in vitro* against Gram-negative bacteria such as *P. aeruginosa* and *E. coli*, Gram-positive bacteria such as *Streptococcus pyogenes* and *Staphylococcus carnosus*, and also demonstrates activity against fungi such as *Saccharomyces cerevisiae* and *C. albicans*. Moreover, one study demonstrated that the DEFB103 has highly potent and salt-resistant antimicrobial activity against *S. aureus* and reduced activity was only observed at supraphysiological concentrations of NaCl (Harder *et al.*, 2001). Most interestingly it was also found to kill the pathogen *Burkholderia cepacia*, that is normally resistant to the activity of antimicrobial peptides (Garcia *et al.*, 2001a).

The fourth β-defensin to be identified, initially termed *DEFB4* and subsequently renamed *DEFB104*, was also identified from genomic sequence analysis (Garcia *et al.*, 2001b). The gene for DEFB104 is composed of two exons separated by a 4.5 kb intron, and encodes a 43 amino acid peptide. *DEFB104* shows a more limited pattern of expression compared to the other human β-defensins, which appear to be diffusely expressed throughout many organs; in contrast expression of *DEFB104* appears to be confined to just the testis, stomach, uterus, thyroid, lung and kidney (Table 1.3). Interestingly, and like *DEFB103* and bovine β-defensins, expression of *DEFB104* was also detected in neutrophils. Expression was up-regulated by treatment of airway epithelia with Gram-positive or Gram-negative bacteria and also in response to phorbol 12-myristate 13-acetate (PMA), but not the inflammatory stimuli that induced expression of DEFB4 (*i.e. DEFB2*) or *DEFB103*. Analysis of the flanking region of the *DEFB104* gene shows the presence of several AP-1 sites, but no NFκB or STAT binding sites were detected. This study also investigated the linkage of the disulfide bridges in

the DEFB104 peptide and further supports the β-defensin arrangement originally seen in BNBD12 (Tang and Selsted, 1993). Functional analysis of DEFB104 demonstrates that the peptide possesses a salt-sensitive, but specific spectrum of antimicrobial activity. The minimum inhibitory concentrations for DEFB104 against Gram-positive bacteria such as *E. coli* and *S. aureus* and the fungi *S. cerevisiae* were in excess of 100 μg/ml. In contrast, DEFB104 showed strong activity against *S. carnosus*, and has particularly potent activity against *P. aeruginosa*, the MIC of 4.5 μg/ml, which makes this peptide one of the most active β-defensin against *P. aeruginosa*.

1.3.4.7 The Murine β-Defensins

As discussed above, mouse neutrophils do not express α-defensins (although Paneth cells in the intestine do). However, five murine β-defensin genes have been identified and reported in some detail, although partial sequences of a further 43 have also recently been reported (Schutte *et al.*, 2002). The reported β-defensin genes are located on chromosome 8A4, which is the syntenic region of the main β-defensins locus, 8p22-23 in humans (Figure 1.6C). The similarities between the different murine β-defensins are shown in Figure 1.6D and in Table 1.4.

The first murine β-defensin to be identified, *Defb1* (also referred to as *mBD-1*), was simultaneously identified by three separate groups based on homology to human *DEFB1*, with which it has 51% identity at the amino acid level (Table 1.4) (Huttner *et al.*, 1997; Bals *et al.*, 1998a; Morrison *et al.*, 1998), and it has since been proposed that Defb1 is an orthologue of DEFB1. As with the other β-defensin, the Defb1 gene contains two exons, the first encodes the

signal sequence and part of the proregion, whereas the second encodes the mature peptide and the remainder of the proregion, separated by a 16 kb intron. The encoded Defb1 peptide is 69 amino acids in length, and constitutive expression has been detected at high levels in the kidney, and at lower levels in the lungs, heart, gut, skeletal muscle and also in purified alveolar macrophages (Table 1.3). Functional analysis of Defb1 has demonstrated that possesses broad-range salt-sensitive antibacterial activity against *P. aeruginosa, E. coli* and *S. aureus,* although the inhibitory effects of salt could be partially abrogated by increased concentrations of peptide (Morrison *et al.,* 1998; Bals *et al.,* 1998a). However, this peptide failed to demonstrate antimicrobial activity against *B. cepacia* (Morrison *et al.,* 1998).

A second murine β-defensin (*Defb2*) has also been reported (Morrison *et al.,* 1999). Expression of *Defb2* was detected in the skin, kidney, lungs, trachea and uterus (Table 1.3). Analysis of the flanking region did not reveal the presence of any regulatory elements, however unlike *Defb1,* expression of *Defb2* was found to be induced by inflammatory stimuli; expression was not detected in primary cultures of murine airway epithelial cells under normal conditions, but following treatment with bacterial LPS expression of Defb2 is up-regulated. The Defb2 gene encodes a 71 amino acid peptide that shows 41% identity to Defb1, and 31% identity to DEFB4. Data relating to the functional characterisation of Defb2 are presented in chapters 3 and 4 of this thesis.

The murine β-defensin 3 (*Defb3,* also called *mBD3*) gene was isolated from mouse lung using primers based on the sequence of a rat β-defensin (Bals *et al.,* 1999). The isolated cDNA exhibited greater similarity DEFB4 (57%) than to DEFB1 (40%) (Table 1.4). Analysis of the gene structure shows that the

presence of the 1.7kb intron separating two exons, a NFκB site is also present in the promoter region of the gene. In contrast to other β-defensins, Defb3 contains an additional amino acid residue between the second and third cysteines. Expression of Defb3 was highest in the salivary glands and epididymis, but expression was also detected in the lung, ovary, liver and brain (Table 1.3). Moreover, expression was induced in the liver and airway epithelia following tracheal installation of *P. aeruginosa*. Functional analysis of the Defb3 peptide showed that it possessed salt-sensitive antimicrobial activity against the Gram-negative bacteria *E. coli* and *P. aeruginosa* that compared favourably to the activity of DEFB4. The authors of this study also propose that there are two types of β-defensin. Type 1 β-defensins such as *DEFB1* and *Defb1* show constitutive expression throughout the epithelia of many mucosal surfaces and possess broad range salt-sensitive antibacterial activity against an array of bacteria. Type 2 β-defensins, such as *DEFB4* and *Defb3*, show inducible expression and possess a narrower range antimicrobial activity, with a tendency to be specific for particular bacteria.

Mouse *Defb4* has also been reported and shown to be expressed in only the oesophagus, tongue and trachea and expression was not induced by inflammatory stimuli (Table 1.3). The gene comprises two exons separated by a 2.4 kb intron, and encodes a 63 amino acid peptide. The Defb4 peptide shares 59% similarity to its putative human orthologue DEFB4 with a much lower level of identity to Defb2 (Table 1.4). However, no functional data are yet available on the activities of this peptide.

The fifth murine β-defensin (*Defb5*) has to date only been reported as a GenBank database entry (AF318068). Recently however, a sixth β-defensin has been cloned from mice. *Defb6* was isolated form a bacterial artificial

chromosome (BAC) and the full-length cDNA subsequently cloned from skeletal muscle. The peptide shows 59% identity to both *Defb3* and *Defb4* (Table 1.4), and is also expressed at detectable levels in the oesophagus tongue and trachea (Table 1.3). Moreover, expression of *Defb6* is induced in the lung by LPS (Yamaguchi *et al.*, 2001). Furthermore, Defb6 showed potent antimicrobial activity against *E. coli* with an MIC of 20 μg/ml. This activity was significantly reduced by high levels of salt. The authors argue suggest that Defb6 plays an important role in maintaining the sterility of skeletal muscle, and also suggest that the narrow pattern of tissue expression of this and other β-defensins may be due to the acquisition of functional adaptations by specific β-defensins to the microenvironment and invading microorganisms in specific organs.

Recently, a novel murine β-defensin was isolated from mouse testis. This defensin related–1 gene (*Defr1*) deviates from the canonical six cysteine motif present in all β-defensins isolated to date (Morrison *et al.*, 2002a). However, surprisingly, Bauer *et al.*, recently reported the crystal structure for a murine β-defensin peptide, *Defb8*, that shows very high levels of identity (98%) to *Defr1* over the entire length of the genomic sequence (Bauer *et al.*, 2001). The *Defb8* gene encodes a peptide that differs from *Defr1* at only three amino acid residues, one of which restores the characteristic six-cysteine motif to Defb8. This suggests that *Defb8* and *Defr1* are sequence variants of the same gene. However, *Defb8* transcripts could not be detected from C57Bl/6N or C577Bl/6J mice by Dr Gillian Morrison who first cloned *Defr1* working in this laboratory. Moreover, the public database of the mouse genome, which is based on genomic sequence of C57Bl/6 mice, also identifies the Defr1 peptide as having only five cysteines, and this further suggests that the Defr1 is the

only form of this gene present. The Defr1 gene contains two exons and an intron of 1.3 kb; analysis of the *Defr1* nucleotide sequence showed greater similarity to *Defb3-Defb7* (similarities greater than 70%) than to Defb1 or Defb2 (similarities less than 50%) (Table 1.4). *Defr1* was most highly expressed in the testis and heart, and at lower levels in the uterus (Table 1.3), however despite the identification of an NFκB site in the promoter region of *Defr1*, intratracheal instillation of *P. aeruginosa* did not induce expression of *Defr1*. Data pertaining to the partial functional characterisation of Defr1 is presented in Chapters 3 and 4 of this thesis.

A recent genome-wide computational search for β-defensin sequences identified a further 27 novel human and 43 new mouse β-defensin second exon sequences in addition to the four human and 6 murine β-defensins described above (Schutte *et al.*, 2002). α-Defensin sequences were not searched for and there may exist a number of α-defensin sequence yet to be identified. This analysis revealed these potential β-defensin genes to be located to five syntenic chromosomal loci. Six novel, potential human β-defensin genes were located to the known β-defensin cluster at 8p22-23; all previous β-defensins had been located to that region, a different cluster was also located to 8p23-22. However this region was not contiguous with the main β-defensin cluster and therefore may represent a separate cluster. More surprisingly, Schutte *et al.* identified a further five β-defensin exon 2 sequences located on chromosome 6p12, and a two other clusters were located to chromosome 20 (20p13 and 20q11.1). In the mouse novel gene sequences were found in region 8A4, where previous β-defensins have been identified, but also in additional clusters on chromosomes 1, 14 and two on 2. Interestingly, these chromosomal locations are syntenic with the regions identified in the human genome; moreover, within each cluster the gene

sequences and organisation were similar, which suggests that each cluster arose from a common ancestor and subsequent divergence of sequence with maintenance of function.

The identification of so many potential β-defensin genes in one study resulted in the reassignment of β-defensin gene names. The human and murine β-defensin genes were reassigned so that the numerical epithet of a particular gene represents the putative orthologous sequence in these two species, in each case the murine β-defensins retain their original name and the human β-defensin gene names are correspondingly reassigned. For example, human *DEFB1* is the putative orthologue of mouse *Defb1* and therefore the names are retained; however, it has been proposed that human *DEFB2* is the orthologue of murine *Defb4*, thus human *DEFB2* was renamed as *DEFB4* and the previously identified human *DEFB4*, for which no murine orthologue has been identified, was renamed *DEFB104*. This classification system (*i.e.* the addition of '10' before the original gene number) is used when there is no clear orthologue identified in the mouse for a given human gene. For example, no murine orthologue has been identified for human *DEFB3* and consequently whereas murine *Defb3* retains that name, *DEFB3* is renamed *DEFB103*. The nomenclature used in the studies presented in this thesis incorporate these names changes.

1.3.4.8 The Evolution of Defensins

The subfamilies of mammalian defensins share some similar characteristics such as their small size, cationicity and the high proportion of cysteines, however, there also appears to be substantial differences between these different families in terms of their patterns of expressions. The question has

been proposed therefore as to whether they have descended from a common ancestral gene by duplication and divergence reviewed in (Hughes, 1999). The similarities between the α- and β-defensins are that the primary structures show a high proportion of cationic residues and six conserved cysteines and that the resulting three-dimensional structures show striking overall similarity. However, the cysteines are the only residues that can be said to be conserved between α- and β-defensins although, the connectivity and spacing of the two defensin subfamilies is different (Figure 1.2) and there are few other conserved residues found in all families of defensins. Thus, it has been argued that the defensin subfamilies are unrelated and have evolved by convergent evolution. However, there is also evidence that the defensins have evolved from an ancestral gene, Liu *et al* (1997) have shown that the α-defensin and β-defensin loci are located within 100-150 kb of each other and propose that this is 'strong direct evidence for evolutionary link; however, linkage in itself is not evidence of a common evolutionary origin. For example, the TAP transporter genes are linked to the major histocompatibility complex (MHC) gene in vertebrates. This link the predates the divergence of bony fishes and tetrapods (Takami *et al.*, 1997); these genes are not evolutionarily linked to MHC class I genes although their products interact functionally. It is possible that the defensin genes originated separately but that their linkage in the genome has been selectively favoured (Hughes, 1997; Hughes, 1999). Notwithstanding these considerations, evidence from structural and sequence analysis appears to support the existence of an evolutionary link between the defensin subfamilies (reviewed in Hughes, 1999). Perhaps the strongest evidence is seen in the region between the third and fourth cysteines, the number of residues in this region (11) is constant – except in a few murine α-defensins, which have 10. Moreover, there are other conserved residues; across many defensins, there is

a glycine and threonine immediately before the fourth cysteine and furthermore, in most cases the residue after sixth cysteine is often positively charged (arginine or lysine).

Gene duplication is a key step in the process by which new genes, such as the defensins, evolve new functions. It has been suggested that gene duplication is followed by a period in which one copy of the gene is functionally redundant and this results in the accumulation and random fixation of neutral or nearly neutral mutations; by chance such mutations may fit a redundant gene for a new function (Ohno, 1973). However, there is evidence from other gene families that duplication and subsequent functional redundancy is not followed by random accumulation of mutations. Rather evidence suggests that positive Darwinian selection leads to acquisition of new functions for the products of the daughter genes (Hughes, 1994). It has also been proposed that duplication of a multifunctional ancestral gene may permit the daughter genes to specialise to one of the functions of the ancestral gene. For example, it is thought that eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), which belong to the ribonuclease superfamily, have evolved from a single EDN-like ancestral gene even though these two genes possess different activities. EDN functions as an antiviral agent via ribonucleolytic destruction of retroviral genomes; in contrast, ECP functions as a membranolytic cationic protein with only low levels of RNase activity. Whilst it is possible that the membranolytic activity of ECP was acquired after duplication, it has also been proposed that the ancestral gene possessed both membranolytic activity and RNase activity and that subsequent duplication allowed one gene to evolve into the ECP gene with potent membranolytic activity (Zhang *et al.*, 1998b).

The theory that α-defensins have evolved by positive selection was tested by analysing the rates of synonymous and nonsynonymous nucleotide substitutions (Hughes and Yeager, 1997). In most proteins, the majority of nonsynonymous substitutions (*i.e.* those changing the encoded amino acid) will be deleterious to protein function and therefore quickly eliminated. In contrast, synonymous substitutions (which do not alter the residue encoded) are selectively neutral (or nearly so). Therefore, in most proteins the rate of synonymous substitutions will be in excess of nonsynonymous substitutions. However, analysis of the sequence of mature α-defensin peptides revealed that the rate of nonsynonymous substitutions exceeded synonymous substitutions. This highly unusual pattern of amino acid substitution suggests that diversification of the α-defensins is selectively favoured. There is also evidence of the same pattern of evolution in bovine and sheep β-defensins (Hughes and Yeager, 1997; Hughes, 1999).

1.3.4.9 Three-Dimensional Structure of β-Defensins

The three-dimensional structures of DEFB1, DEFB2, DEFB103, Defb7 and Defb8 have recently been reported (Hoover *et al.*, 2000; Harder *et al.*, 2001; Bauer *et al.*, 2001; Schibli *et al.*, 2002). All the β-defensins have shown similar structures. The peptides consists of three anti-parallel β-strands similar to that of the α-defensins, however all human and murine β-defensins analysed to date have an α-helical structure at the N-terminus (Figure 1.7) that is absent from α-defensins and bovine β-defensins (Hill *et al.*, 1991; Zimmermann *et al.*, 1995). Also in contrast to α-defensins, many of the

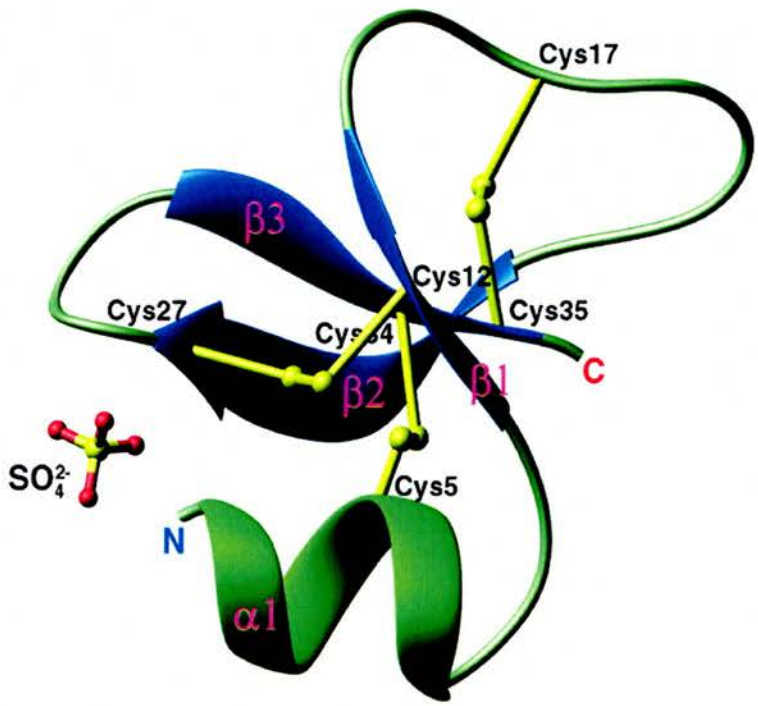

Figure 1.7: Schematic Diagram of the Three-Dimensional Crystal structure of a DEFB1 monomer. The α-helix is represented in green and the three anti-parallel β-sheets (β1, β2, β3) are shown in blue. The crystal structure of the DEFB4 monomer showed strking similarity to the DEFB1 structure. Figure taken from (Hoover *et al.*, 2001).

β-defensins analysed appear to lack a defined amphipathic structure, with no clearly identifiable pattern of charged and hydrophobic residues on the surface with the accommodation of a wide range of different amino acid residues at most sequence positions (Bauer *et al.*, 2001). It has been proposed that this feature of β-defensin structure suggests that β-defensins antimicrobial activity is mediated by the 'carpet model' rather than through the formation of stable functional barrel-stave pores (Hoover *et al.*, 2000). The structures of some β-defensins show a capacity for oligomerisation. For example, DEFB4 monomers form dimers via interaction of the first β-sheets and they also show a quaternary arrangement of four dimers to produce a higher order structure of DEFB4 molecules (Hoover *et al.*, 2000). The authors propose that the octomer is the form that interacts with bacterial membranes. Whilst DEFB1 dimers were observed in the crystal structure, there was no evidence however of higher order structures such as the DEFB4 octomer (Hoover *et al.*, 2001). Separate studies have suggested that whilst DEFB103 forms amphipathic dimers via the second β-sheet, DEFB4 and DEFB1 exist only as monomers. The authors suggest that the detection of DEFB4 the higher order structures identified in the Hoover *et al.* study (2000) is due to the high peptide concentration required for crystallisation of the peptide (Bauer *et al.*, 2001; Schibli *et al.*, 2002). Interestingly, it has also been shown that DEFB103 can generate ion channel activity the membrane of *Xenopus* oocytes, suggesting that DEFB103 may function by the pore-forming mechanism (Garcia *et al.*, 2001a).

1.3.4.10 Additional Functions of β-defensins

As with the α-defensins, it has been suggested that the β-defensins possess additional cellular functions other than their direct antimicrobial activity. Several studies have suggested that β-defensins may have a growth inhibitory or eradicative role at epithelial surfaces. Incubation of DEFB1 with the mouse tumour cell line NIH 3T3 demonstrated that the β-defensin was cytotoxic and the authors propose that DEFB1 may play a role in eradicating cells from epithelia in inflammatory processes (Zucht *et al.*, 1998). However, it remains unclear whether the observed cytotoxicity was via necrotic or apoptotic mechanisms. Moreover, an analysis of gene expression in renal cell carcinomas found that the expression of *DEFB1* showed a seven-fold cancer-specific down-regulation compared to benign tissue. This suggests that This suggests that down-regulation of DEFB1 expression may be advantageous to cell growth (Young *et al.*, 2001). Furthermore, it has also been proposed that β-defensins may play a role in cell differentiation (Frye *et al.*, 2001). When the expression of *DEFB1* was monitored in relation to the differentiation and proliferation of human HaCaT cells, it was found that *DEFB1* expression was low during proliferation but induced upon differentiation. Moreover, *DEFB1* was overexpressed in keratinocytes and analysis of the marker keratin 10 found its expression to be greatly up-regulated in the presence of high levels of DEFB1. These data suggest that DEFB1 may promote differentiation processes in keratinocytes. In another recent study DEFB4, but not DEFB1, was shown to induce prostaglandin D_2 synthesis and degranulation in rat peritoneal mast cells, this activity was also sensitive to pertussis toxin which suggests that the effects are mediated via a G protein coupled receptor (Niyonsaba *et al.*, 2001).

Perhaps the most studied area of β-defensin function other than the spectra of antibacterial activity is their ability to act as chemokines for immune related cells. Previous studies have shown that DEFB4 can induce migration of immature CD34+ progenitor derived dendritic cells and $CD4^+$ T-cells *in vitro* at nanomolar concentrations (Yang *et al.*, 1999). However, in contrast to HNP-1 and -2, which attract the $CD4^+/CDRA^+$ naïve subset of T-cells, DEFB4 was chemotactic for the $CD4^+/CD45RO^+$ memory subset of T-cells. Mature dendritic cells, naïve T-cells and monocytes did not migrate in response to DEFB4. This migration was found to be pertussis toxin-sensitive, again suggesting the role of a G protein-coupled seven-transmembrane domain receptor. Moreover, migration of HEK-293 cells transfected with the chemokine receptor CCR6 was induced by DEFB1 and DEFB2 at similar concentrations as dendritic cells and CD4 T-cells. In contrast, cells transfected with CXCR4, CCR1 and CCR5 (the only other chemokine receptors expressed on $CD34^+$ dendritic cells) did not migrate suggesting that β-defensins act solely through CCR6. A recent study has demonstrated that DEFB4 is also chemotactic for mast cells, and it is thought that activation is mediated via a different receptor (Niyonsaba *et al.*, 2002). DEFB103 has been shown to be chemotactic for monocytes but not for neutrophils (Garcia *et al.*, 2001a), this suggests that different β-defensins do not exhibit the same pattern of chemotactic activity and that as with antibacterial activity β-defensins may have developed a certain degree of specialisation. DEFB104 also possesses chemotactic activity for monocytes, but not for eosinophils or for neutrophils (Garcia *et al.*, 2001b). Another study has shown that a murine Defb2 or Defb3 peptide fused to the lymphoma sFv antigen was chemotactic for immature dendritic cells, but not mature dendritic cells (Biragyn et al., 2001); this study however did not analyse the chemotaxis of Defb2 or Defb3

peptides alone. Although, the sFv antigen alone was not chemotactic, suggesting that the cell migration was due to Defb2 and Defb3. This thesis presents novel data of the chemoattractant activity of Defb2 and Defr1. Chemotaxis and the different immune cell types pertinent to this thesis are discussed in greater detail in section 1.7.

1.4 Other Antibacterial Peptides

1.4.1 Cathelicidins

Cathelicidins constitute another main family of antibacterial peptides of mammals (Lehrer and Ganz, 2002a). Cathelicidins consist of a putative N-terminal signal peptide, a highly conserved cathelin (*cathe*psin *L in*hibitor)-like domain in the middle of the molecule and an less conserved antibacterial C-terminus (Figure 1.8).

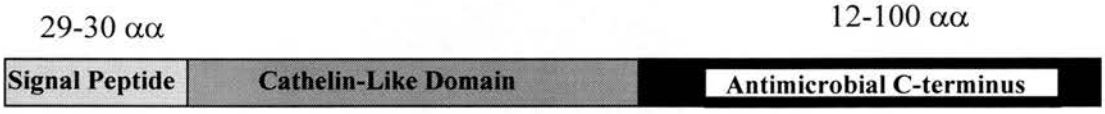

Figure 1.8: The Conserved Structure of Cathelicidin Peptides. The signal peptide is cleaved prior to further processing and the cathelicidin domain shows a highly level of conservation among the different cathelicidin peptides; however, the antimicrobial C-terminus shows high-levels of diversity sequence of secondary structure. αα represents amino acids. Diagram adapted from Yang *et al.*, (2001).

Cathelicidins have been identified in many mammalian species, including cattle (e.g., indolicidin and bactenecins), mouse (e.g., mCRAMP) and pigs, where they are particularly abundant (for example the proline rich peptide of 39 residues, PR-39 and also the protegrins) reviewed in (Ganz and Weiss, 1997). To date, however, only one cathelicidin peptide has been identified in

humans, called hCAP18/LL37 (Bals *et al.*, 1998c) (Agerberth *et al.*, 1995; Cowland *et al.*, 1995; Larrick *et al.*, 1995). The cathelin-like prepropeptide, hCAP18 is cleaved off by neutrophil elastase to release the antimicrobial C-terminus peptide called LL37. Cathelicidins are primarily expressed in the granules of neutrophils and released upon stimulation, however LL37 is also expressed by epithelial cells such as in the lung and also by monocytes and T-cells (Agerberth *et al.*, 2000). The C-termini of cathelicidins vary considerably across the animal species in both sequence and structure (Yang *et al.*, 2001). For example, LL37 and rabbit CAP18 have an α-helical structure, whereas others such as PR39 and the bovine bactenecins are show a polyprohelical structure, and porcine protegrins have β-sheet structures. Cathelicidins also possess a wide range of functions, including salt-sensitive antibacterial activity against both Gram-positive and Gram-negative bacteria and *Candida albicans* (Zanetti *et al.*, 1995; Cho *et al.*, 1998; Turner *et al.*, 1998). They have also been shown to have LPS-neutralising activity (Hirata *et al.*, 1994a; Hirata *et al.*, 1994b; Larrick *et al.*, 1994), to be chemotactic for mast cells, T-cells and phagocytes (Agerberth *et al.*, 2000; Yang *et al.*, 2000b ; Yang *et al.*, 2000c ; Niyonsaba *et al.*, 2002).

1.4.2 Histatins

Histatins are small histidine-rich peptides that are present in human saliva and display moderate activity again *C. albicans* particularly under acidic conditions and low salt concentrations (Tsai and Bobeck, 1997). Interestingly, it is thought that Histatins are not membranolytic and instead act by binding to a specific 67 kDa *C. albicans* protein (Edgerton *et al.*, 1998).

1.4.3 Granulysins

Granulysin is a peptide that belongs to the saposin-like protein gene family and is found in the granules of $CD8^+$ T-cells and natural killer (NK) cells (Stenger *et al.*, 1998). Studies with recombinant human granulysin demonstrated that the peptide had antimicrobial activity against a broad range of microorganisms, including *Mycobacterium tuberculosis*.

1.4.4 Cathepsin G

Cathepsin G is a neutral serine proteinase that is primarily present in the azurophilic granules of neutrophils and to a lesser extent in a membrane-bound form (Chertov *et al.*, 1997). Additional functions that are ascribed to Cathepsin G includes the activities seen with other antimicrobial peptides such as antibacterial activity, neutrophil and monocyte/macrophage chemotactic activity, but Cathepsin G also stimulates T-cell proliferation and production cytokines such as interleukin-1, tumour necrosis factor-α (TNFα) and interferon-γ (IFN-γ) (Yang *et al.*, 2001; Travis, 1997).

1.4.5 CAP37/Azurocidin

The human cationic antimicrobial peptide 37 (CAP37) was isolated from neutrophil granules as a component of the oxygen–independent antimicrobial activity of phagocytes (Shafer *et al.* 1984) and is also known as azurocidin (Gabay *et al.*, 1989). The peptide is synthesised as a 351 residues precursor and processed to give a mature peptide of 222 amino acids. It has strong homology to serine proteases such as neutrophil elastase and, whilst it

is too large to fit Scott's and Hancock's (2000) definition of a cationic antimicrobial peptide (12 to 50 amino acids), it warrants consideration here as it possesses no enzymatic activity, but exhibits a range of activities similar to other antibacterial peptides (Yang et al., 2001). Apart form its antibacterial activity it is also chemotactic for monocytes/macrophages (Chertov *et al.*, 1997) and T-cells (Chertov *et al.*, 1996) and also binds to and neutralises LPS (Pereira *et al.*, 1993).

1.5 Bacterial Resistance Mechanisms to Cationic Antibacterial Peptides

As discussed above the initial target of the cationic antimicrobial peptides are the LPS, lipoteichoic acids (LTA) and anionic membrane phospholipids, such as phosphatidylglycerol. Despite these targets being of fundamental importance, some species of bacteria have developed mechanisms to alter these structures and thus reduce the attraction of cationic peptides for them reviewed in (Lehrer and Ganz, 2002b; Peschel and Collins, 2001).

S. aureus and *S. xylosus* tolerate high concentrations of antimicrobial peptides. Laboratory-generated mutants were identified that displayed increased sensitivity. Subsequent analysis revealed that the LTA of these strains lacked D-alanine, and therefore the cells display an increased negative surface charge (Peschel *et al.*, 1999). The mutated genes were found to be in the *dlt* operon and this complex is responsible for the transportation of D-alanine and incorporating it into the LTA. The LTA backbone is highly charged and the incorporation of D-alanine reduces the net negative charge and consequently the mutant *S. aureus* in which the LTA contains no detectable

alanine has a higher net negative surface charge and therefore a greater binding capacity for cationic peptides (Peschel *et al.*, 1999).

The *mprF* gene of *S. aureus* also confers some degree of resistance to antimicrobial peptide on *S. aureus* (Peschel *et al.*, 2001). A *mprF* mutant strain *S. aureus* was killed considerably faster by human neutrophils and also exhibited reduced virulence in mice compared to the wildtype. This suggests that *S. aureus* resistance to defensins may play a key in the pathogenicity of this bacterial species. The MprF gene product covalently modifies the membrane phosphatidylglycerols with L-lysine, which has the effect of reducing the net negative charge of the membrane surface. Moreover, similar genes have been identified in *Mycobacterium tuberculosis, Enterococcus faecalis* and *P. aeruginosa.*

While modification of the cell membrane will reduce its negative charge and therefore the attraction of cationic peptides bacteria such as *Staphylococcus epidermidis* possess a membrane bound transporter that ejects the peptide from the membrane once they have inserted into the lipid bilayer via ATP hydrolysis. However, this gene complex is used to protect *S. epidermidis* from self-encoded antibacterial peptides such as epidermin and may not generate resistance to mammalian antibacterial peptides such as defensins. Furthermore, the naturally occurring staphylococcal plasmid pSK1 has been shown to confer resistance to HNP-1 and also the protegrin-1; this plasmid encodes the drug exporter QacA, a proton motive force-dependent efflux pump which transports peptides from the cell (Kupferwasser *et al.*, 1999). Other members of this family of pumps are the Bmr proteins of *E. coli* and he EmrB protein of *B. subtilis* (Peschel and Collins, 2001). Other bacterial

species such as *P. aeruginosa, Enterococcus faecalis* and *Mycobacterium tuberculosis* possess several extracellular proteases that attack matrix proteoglycan and cause the release of dermatan sulfate an anionic molecule that binds to and inhibits the activity of cationic antimicrobial peptides (Schmidtchen *et al.*, 2001). Resistance in *Salmonella typhimurium* to cationic peptides is also provided by PhoP-PhoQ proteins, which are though to mediate resistance via the effector genes *pmbrA-pmbrB*. This results in alterations to the structure of the LPS, which renders *S. typhimurium* less sensitive to the activities of cationic peptides (Fields *et al.*, 1989). Moreover, PhoP-PhoQ have also been implicated in the observed resistance of *P. aeruginosa* to cationic antimicrobial peptides (Macfarlane *et al.*, 2000).

1.6 A Role for β-Defensins in Cystic Fibrosis

As was discussed earlier it has been proposed that the salt-sensitive activity of β-defensins may play a role in the pathogenesis of cystic fibrosis, although this theory remains controversial and there is a paucity of supporting data.

1.6.1 Pathology and Aetiology of Cystic Fibrosis

Cystic fibrosis is caused by a dysfunction of ion transport across epithelial due to a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR). The disease affects many different systems: the gastrointestinal tract, pancreas, lung reproductive tract, liver and sweat ducts are all altered in CF patients.

1.6.2 Cystic Fibrosis Lung Disease

The most serious clinical manifestation of CF is lung disease, which causes 90% of morbidity in CF individuals. CF lung disease is characterised by chronic bacterial colonisation with repeated acute infection by a distinct flora of bacteria. These infections induce prolonged inflammatory responses that produce permanent lung damage. This establishes a vicious cycle of infection, inflammation and airway damage. Typically, this results in progressive bronchiectasis (abnormal and irreversible dilation of bronchioles), respiratory failure and death.

The progress of CF lung disease sees an age dependent 'evolution' of the bacterial pathogens that colonise the lung. Infections are established in infants by *Staphylococcus aureus* and *Haemophilus influenzae,* but in later years *Pseudomonas aeruginosa* is the dominant microorganism. Moreover, in some cases the opportunistic pathogen *Burkholderia cepacia* may also colonisation the lung.

1.6.3 From CFTR Mutation to CF Lung Pathology

It is still unclear how a defect in CFTR leads to lung infection by organisms that are not normally an issue in human health, or to the excessive inflammatory response. Several hypotheses have been proposed to explain this; CFTR mutation may permanently alter the lung environment making it susceptible to colonisation and infection, or there may be a more direct effect of CFTR mutation (e.g. preceding airway inflammation). The differing theories need not be mutually exclusive and it is probable that CF lung disease will be caused by a multiplicity of factors.

1.6.3.1 The Airway Surface Liquid

Two theories, which attempt to explain why the CF airway is prone to infection, are based on alterations in the fluid that the lines the airways, the airway surface liquid (ASL). ASL is composed of two phases, gel and sol (perciliary), and contains a wide range of macromolecular products, including glycoproteins, proteoglycans, lipids, defence molecules, DNA and actin (reviewed in Pilewski and Frizzell, 1999). ASL covers both the distal and proximal airways, though the thickness is reported to be relatively constant (5-10 μm) and move up the airways by cilia action at the rate of between 1.0 to 10 mm/min; thereby the proximal airways may receive liquid from distal surfaces. ASL can also be added by the submucosal glands, although the actual contribution of submucosal glands to ASL is unknown (Boucher, 1994).

1.6.3.2 The High Salt Theory

This theory proposes that the ASL has an increased NaCl concentration and this serves to inhibit the action of part of the lungs innate defence system. This theory is based on work by Zabner and colleagues (1998) who demonstrated, using a primary culture system, that CFTR is required for maximal absorption of salt and water; with large amounts of water on the apical surface both water and salt are absorbed. With tiny amounts on the surface, salt is absorbed in excess of water; thus according to this model normal ASL is hypotonic to blood plasma. The reduction in Cl^- absorption would result in lower Na^+ absorption and water, which would be absorbed by osmosis, is prevented from falling below the level of the cilia by capillary

forces generated by the cilia. Therefore, in this model, CFTR mutation leads to lower Na^+ and Cl^- absorption and as water absorption is prevented, consequently the ASL of CF patients would have an elevated NaCl concentration (Figure 1.9A).

Zabner and colleagues (1998) found ASL to be hypotonic and ASL from cultured CF cells to have an increased NaCl concentration; they demonstrated that Na^+ concentrations of 50 mM in normal cells with 100 mM in CF cells and the Cl^- concentration of normal cells to be 37 mM, elevated to 90 mM in CF cells. An earlier study demonstrated that elevated NaCl levels disabled the lungs innate antibacterial activity (Smith *et al.*, 1996). *P. aeruginosa* placed on the epithelia of normal cells in primary culture were killed, but bacteria placed on the CF cells multiplied; the antibacterial activity could be restored to CF epithelia with the expression of CFTR. Furthermore, this antibacterial activity was shown to be a property of ASL, and that it was lost when the NaCl of normal cells was increased and could be restored to CF ASL when the salt concentration was lowered (Smith *et al.*, 1996). Work by Goldman and colleagues suggested that this inactivated component of the lungs defence system could be the β-defensin DEFB1. Using a human bronchial xenograft model, the concentration of Na^+ and Cl^- was found to be 83 mM in ASL from xenografts, but in CF ASL Na^+ was found to be 178 mM and Cl^- was 172 mM. Furthermore, it was demonstrated that anti-sense oligonucleotides designed to the sequence of *hBD-1* ablated the ability of normal xenografts to kill bacteria. Importantly it was also shown that DEFB1 has salt sensitive antibacterial killing and may therefore be disabled in the CF lung, thereby predisposing it to infection (Goldman *et al.*, 1997).

1.5.3.3 The Mucus Dehydration Theory

While the high salt theory remains an attractive hypothesis the majority of studies fail to find evidence for elevated salt levels in the ASL of CF patients. For example, an important and elegant study by Jayaraman *et al.* (2001) found no significant difference between the NaCl concentrations of the ASL of CF individuals compared to non-CF individuals, however they did report an elevated viscosity of the ASL, supporting the classical or mucus dehydration theory of CF pathogenesis. The classical view of the CF lung is of excessive production of dehydrated mucus clogging up the airways and impeding the mucociliary escalator that is designed to remove bacteria and other particulate matter from the lungs to the mouth and nostrils where it can be removed. Bacterial products and the inflammatory response would stimulate mucus secretion further, thus exacerbating the situation leading to obstruction of the airways; this would establish the vicious cycle described earlier.

Crucial to this model is the large disparity between the surface area of the distal and proximal airways ($70m^2$ and $60cm^2$, respectively) and that the depth of ASL remains constant between the two regions (Boucher, 1994). This implies that there must be absorption of large amounts of liquid as the ASL is moved up the airways, estimations put this volume at approximately 700ml (Pilewski and Frizzell, 1999). In normal airways this would be driven by absorption of Na^+ through ENaC, Cl^- would follow via CFTR and paracellular routes and water would be absorbed by osmosis. It has been proposed that CFTR also serves to suppress the activity of the epithelial sodium channel (ENaC) and thus in the absence of CFTR there would be misregulation of ENaC resulting in excessive Na^+ absorption and thus an

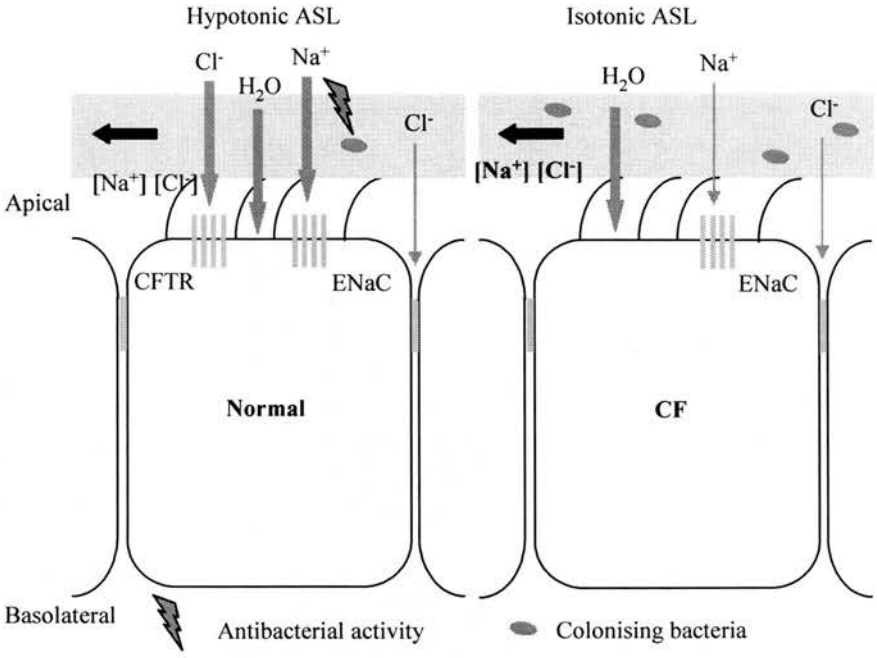

Figure 1.9A: The High Salt Theory. In the absence of CFTR there is greatly reduced Cl^- absorption with minimal absorption occurring via the paracellular route. Consequently Na^+ absorption is reduced and thus the concentration of NaCl in the ASL is raised which disables the antibacterial activity, thus predisposing the airway to infection. Adapted from Guggino, 1999.

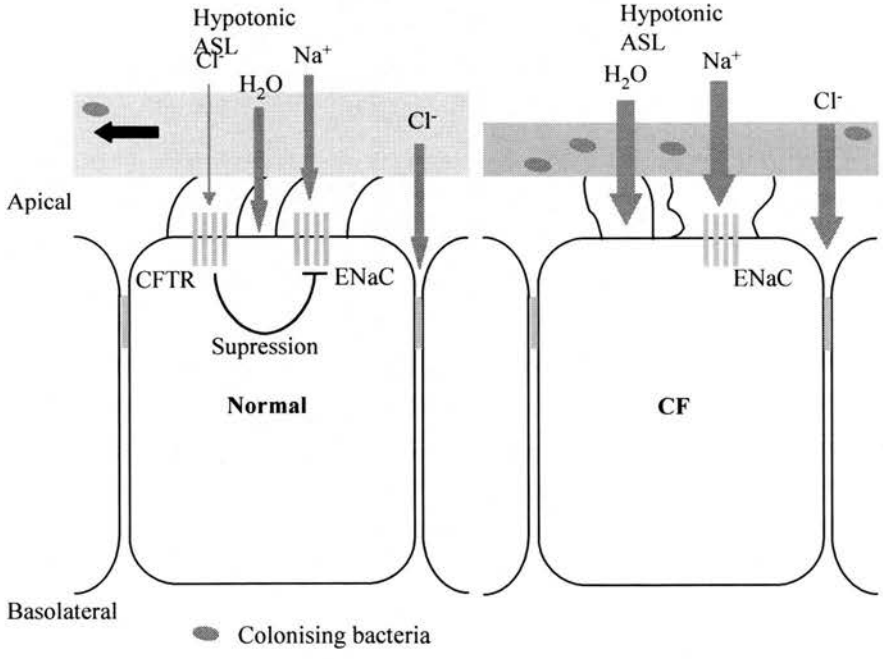

Figure 1.9b: The Dehydrated Mucus Theory. Na^+ absorption is greater in CF cells due to misregulation of ENaC, Cl^- absorption via the paracellular route and water absorption (via osmosis) are increased leading to insipissation of the mucus, which disables mucociliary clearance. Adapted from Guggino, 1999.

increased level of water absorption (Figure 1.9b) (Matsui *et al.*, 1998). This would result in dehydration of the mucus, and if the sol layer drops below the level of the cilia – as it was observed to do in the Matsui model system, the mucociliary clearance system would be impaired.

1.6.3.4 Other Models for CF Lung Pathogenesis

Many other theories have been proposed to account for the pathogenesis of CF lung disease. It has been suggested that acts as a specific cellular receptor for *P. aeruginosa* via the oligosaccharide core of its lipopolysaccharide (LPS) and this leads to internalisation. In CF, this process would be impaired and therefore bacterial clearance reduced (Pier, 2000). According to the bacterial adherence theory CF pathogens adhere better to CF epithelia than to normal epithelia. It has been suggested that this is due to a higher proportion of asialylated glycolipids present on CF epithelia compared to normal epithelia (Saiman and Prince, 1993). Furthermore, further studies have suggested that the loss of CFTR leads to defective acidification of intracellular organelles, which results in the altered levels of asialylated glycolipids (Barasch *et al.*, 1991).

1.7 Microbiology of the CF Lung

As discussed above chronic bacterial colonisations and repeated bacterial acute infections are a major symptom of CF. Interestingly, these infections are by a narrow spectrum of bacteria that are not normally a health threat. Moreover, the progress of CF lung disease sees an age-dependent 'evolution' of the bacterial pathogens that colonise the lung. Infants with CF frequently suffer infection by *Staphylococcus aureus*, which dominates in the first two

years of life (often before the onset of overt clinical symptoms), and this is followed by *H. infleunzae*. The next stage is characterised by the predominance of *P. aeruginosa*, with a third of CF individuals infected by the age of 5 years (Grimwood, 1997), rising to 90% of CF adults (Hutchison and Govan, 1999). To add to the difficulties in treating CF, multidrug-resistant strains from *Burkholderia cepacia* complex have recently emerged as a new CF pathogen (Gilligan, 1991). The surprising feature of CF lung disease is that these bacteria do not normally colonise the lung, although they may occasionally cause concern in immunosuppressed patients; for example, *B. cepacia* is more commonly regarded as a plant pathogen. Colonisation of the lung by mucoid strains of *P. aeruginosa* is highly problematic and as they are rarely cleared from the lung and represent a poor prognosis.

1.7.1 *Staphylococcus aureus*

The organism first recognised as a serious CF pathogen in the lungs of young children was *S. aureus* (Anderson, 1949), although the use of aggressive antibiotic therapy had reduced the incidence of *S. aureus* infections coincident with an increase in infections with *P. aeruginosa*. However, the development of antibiotic resistant strains means that S. *aureus* remains a major human pathogen. Reports have suggested that as many as 85% of community acquired *S. aureus* infections are penicillin resistant, and of particular concern was the emergence of methicillin-resistant *S. aureus* (MRSA) in the 1980s. The resistance to methicillin is conferred by the *mecA* gene and resistance to other antibiotics has also been linked to *mecA* (Cohen., 1986; Kreiswirth *et al.*, 1993; reviewed in Hutchison and Govan, 1999). Moreover, *S. aureus* remains a pathogen that predominates in the lungs of CF patients in the first few

years of life. Indeed, *S. aureus* is distinguished by a particular capacity to cause both community and hospital acquired infections and it remains one of the most frequent human pathogens causing severe morbidity and life-threatening diseases of various organ systems (Peschel and Collins, 2001).

S. aureus is a Gram-positive, non-motile, non-spore-forming coccus. It is a human commensal, commonly isolated form the upper respiratory tract, nasal passages and the skin of healthy individuals, but is also an important pyogenic organism and produces a number of haemolysins and enterotoxins (Coia *et al.*, 1992; Hutchinson and Govan, 1999). Moreover, *S. aureus* produces teichoic acid and exopolysaccharide that appear to play a role in adherence to respiratory epithelium in CF patients (Aly *et al.*, 1987). The precise role of these and other virulence factors in pulmonary colonisation and subsequent evasion of immune clearance remains unclear. Several previous studies have shown that *S. aureus* is susceptible to the activity of β-defensins (Singh *et al.*, 1998; Morrison *et al.*, 1998). However, as discussed in section 1.5, *S. aureus* has also developed resistance mechanisms to mammalian and bacterial antimicrobial peptides, which can broadly protect against a range of cationic peptides (Peschel *et al.*, 1999; Peschel *et al.*, 2001; reviewed in Peschel and Collins, 2001).

Infections with the Gram-negative *Haemophilus influenzae* are also common in young CF patients, however, the role of this bacterium in the evolving pathogenesis of CF is controversial and its importance may be underrated as many clinics do not routinely treat patients colonised with this organism (Govan and Nelson, 1992).

1.7.2 *Pseudomonas aeruginosa*

Early studies of the bacterial flora of the CF lungs suggested that *P. aeruginosa* played only a minor role in the microenvironment. However, it appears that the introduction of antibiotic therapy may have had the effect of reducing the effect of *S. aureus* infections and increasing the incidence of the more antibiotic resistant *P. aeruginosa.* Subsequently, this bacterium has been recognised as the most important CF lung pathogen, responsible for chronic bronchopulmonary infection and progressive lung disease (Govan and Nelson, 1992). After the first two years of life, *P. aeruginosa* infections begin to predominate in the CF lung. A third of CF individuals are infected by the age of 5 years, and this rises to 90% of CF patients in adulthood (Grimwood *et al.*, 1997; Govan and Nelson, 1992).

P. aeruginosa is a Gram-negative, motile, non-spore-forming, non-capsulate bacillus widely found in soil and water and is generally acknowledged as an opportunistic pathogen that causes a wide range of infections in immunocompromised patients. The most striking feature of this colonisation is that the infection remains confined to the lungs and that the organism undergoes considerable phenotypic change. Most notable is the emergence of mucoid variants due to excessive production of the exopolysaccharide alginate these strains are unique to CF and practically diagnostic of the disease (Doggett *et al.*, 1964; Govan and Deretic, 1996). *In vivo*, the mucoidy *P. aeruginosa* grows within this alginate that is composed of the highly charged exopolysaccharide, host DNA and host mucins, and which serves to protect the bacteria from antibiotics and phagocytosis (Anwar *et al.*, 1992; Govan and Harris, 1986; Simpson *et al.*, 1988). The *P. aeruginosa* infection is

established by non-mucoidy, planktonic strains and then due to environmental pressure these organisms convert to the mucoidy phenotype, which predominates during the chronic lung infection in CF patients and a single strain appears comes to dominate. Moreover, once established, growth by the mucoid *P. aeruginosa* is hardly ever cleared and represents a poor prognosis (Gilligan, 1991).

P. aeruginosa is naturally resistant to many antibiotics due to the relatively impermeable OprF porin in the outer membrane, and active efflux system and a β-lactamase enzyme (reviewed in Hancock, 1998). However, several studies have demonstrated that it is susceptible to the activity of β-defensins (Goldman *et al.*, 1997; Singh *et al.*, 1998; Morrison *et al.*, 1998).

1.7.3 *Burkholderia cepacia complex*

During the 1980s, another bacterial pathogen emerged as a significant CF lung pathogen, the first reported isolation of *Burkholderia cepacia* from lungs of a CF patient was in 1972 (Ederer and Matsen, 1972). The prevalence of *B. cepacia* infection in CF has risen from 10% in the 1970s (Isles *et al.*, 1984), to the current level of approximately 20% (Hutchison and Govan, 1999). However, there exists considerable clinic-to-clinic variation, with an incidence of 30-40% in some clinics where there was significant contamination between patients, and reaching as high as 70% in some large clinics (Govan *et al.*, 1996). Moreover, *B. cepacia* has also been found to cause macerated hyperkeratonic foot lesions in otherwise healthy marines living in swampy conditions (Taplin, 1971).

B. cepacia is a Gram-negative, non-sporing, aerobic, motile bacillus that is found in soil and water. It was initially described as a plant pathogen from a study of the soft rot of onions (Burkholder, 1950) and not immediately recognised as a human pathogen. *B. cepacia* has a large genome of at least 7 Mb composed of as many as three megabase-sized replicons (Rodley *et al.*, 1995). *B. cepacia* has been shown to produce several virulence factors (reviewed in Hutchison and Govan, 1999). These include a haemolysin that produces pores in the membrane of erythrocytes, at least two proteases, which show similarity to *P. aeruginosa* elastase, lipases and a catalase. However, only the endotoxin has been shown to have a definitive role in the pathogenesis of *B. cepacia* (Hughes *et al.*, 1997; Hutchison *et al.*, 1998). *B. cepacia* also possesses a giant cable pilus that has strong affinity for mucin binding and may serve to anchor bacteria to host lung epithelium (Sajjan *et al.*, 1995).

B. cepacia is notoriously resistant to the action of antibiotics, antimicrobial peptides (Hancock, 1997a; Baird *et al.*, 1999) and no effective antibiotic therapy is yet available. Indeed one study demonstrated that *B. cepacia* could use penicillin G as the sole carbon source (Beckman and Lessie, 1979). Strains of this multiresistant bacterium constitute a threat to both CF patients and individuals with chronic granulomatous disease, and mouse models of CF have been shown to have an increased susceptibility to *B. cepacia* (Sajjan *et al.*, 2001). This multiresistance of *B. cepacia* can be attributed to the development of several different resistant mechanisms (reviewed in Hutchison and Govan, 1999). A β-lactamase gene that encodes a 31-kDa protein that confers resistance to several penicillins such as imipenem and carbenicillin has been

identified (Trepanier *et al.*, 1997); an efflux pump homologous to the *oprM* efflux pump of *P. aeruginosa* has also been described (Burns *et al.*, 1996).

Until 1992, *B. cepacia* was actually classified as a *Pseudomonas* and referred to as *P. cepacia*; however at this time it, and six other species belonging to the *Pseudomonas* rRNA group II, were reassigned to the new genus *Burkholderia* (Yabuuchi *et al.*, 1992). However, there was a marked heterogeneity among several *B. cepacia* strains isolated from both clinical and environmental sources and the different strains were subsequently subdivided into at least five genomic species (genomovars) based on DNA-DNA and DNA-rRNA hybridisations, fatty acid content and whole-cell protein extract analyses (Vandamme *et al.*, 1997; reviewed in Coenye *et al.*, 2001a). Since *B. cepacia* genomovar I contains the type-strain it retains the formal name *B. cepacia*; however, *B. cepacia* genomovar V was recognised as the previously identified *B. vietnamensis* and genomovar II was renamed *B. multivorans* (Vandamme *et al.*, 1997) and recently genomovar IV was reclassified as a new species named *B. stabilis* (Vandamme *et al.*, 2000). Subsequent analyses identified two additional members of the *B. cepacia* complex. Firstly, *B. cepacia* genomovar VI, it is possible to phenotypically distinguish this organism from all other *B. cepacia* complex members except for *B. multivorans* (Coenye *et al.*, 2001b), and *B. cepacia* genomovar VII, which has been named *B. ambifaria* and include isolates form clinical and environmental specimens, such as strains from CF patients as well as several well-characterised biocontrol strains (Coenye *et al.*, 2001c). There have been further recent additions to the *B. cepacia* complex; *B. anthinia* has been recognised as genomovar VIII and *B. pyrrocinia* as genomovar IX (Vandamme *et al.*, 2002). In the absence of clear-cut biochemical tests to distinguish genomovars III and VI from *B. cepacia*

genomovar I and *B. multivorans* (genomovar II), these genomovars have not been formally named. However, it has have recently been proposed that *B. cepacia* genomovar III should be renamed as *B. cenocepacia* (Vandamme *et al.*, 2002).

Studies of *B. cepacia* complex infections in CF patients suggested that most infections are actually caused by *B. multivorans* (genomovar II) and *B. cenocepacia* (genomovar III) strains (Agodi *et al.*, 2001; LiPuma *et al.*, 2002). Indeed genomovar III colonised patients were at higher risk for complications and mortality (Aris *et al.*, 2001). Moreover, genomovar III strains may replace other *B. cepacia* complex strains in the CF lung (Mahenthiralingam *et al.*, 2001) and most outbreak strains also belong to genomovar III (Speert *et al.*, 2002). Furthermore, other studies have indicated that *B. cepacia* infection can be spread form patient to patient, and this has lead to isolation of infected patients. As indicated above, the transmissibility of *B. cepacia* is strain dependent and outbreaks result from epidemic strains, such as the Edinburgh-Toronto (Electrophoresis type 12 (ET/12)) strain. This bacterium was isolated from the sputum of 33 of the 60 cases of *B. cepacia* colonisation in Edinburgh and Manchester CF clinics between 1986 and 1992 (Govan *et al.*, 1993; Govan and Deretic, 1996). This strain often referred to as *B. cepacia* J2315, is one of the most resistant of the *B. cepacia* strains to conventional antibiotics (Nzula *et al.*, 2002) and used in this thesis to assess the activity of novel β-defensins. In this thesis however the proposed new name *B. cenocepacia* is used. Previous studies have shown that strains from the *B. cepacia* complex are resistant to the activity of cationic peptides such as β-defensins (Morrison *et al.*, 1998; reviewed in Hancock, 1997a). However the recently reported DEFB103 was shown to have antibacterial activity against

B. cepacia with an MIC of 6.6 μg/ml, although this remains the only report of a β-defensin active against *B. cepacia* (Garcia *et al.*, 2001a).

1.8 Aims of this Thesis

The aims of this thesis were to investigate the antibacterial and chemoattractant activities of murine β-defensins.

The murine β-defensin Defb2 was isolated recently by Dr Gillian Morrison working in this laboratory. However, its antibacterial activity has not been assessed and therefore on of the aims of this project is to assess the antibacterial activity against a range of Gram-negative and Gram-positive bacteria. This project was established, at least in part, to investigate the potential role of β-defensins in the pathogenesis of cystic fibrosis (CF), and therefore many of the bacteria used in this thesis are CF pathogens. The antibacterial activity of the novel murine β-defensin Defr1, which lacks the first canonical cysteine present in all β-defensin identified to date, is also assessed.

Several studies have suggested that human β-defensins possess chemotactic activity *in vitro*; it has also been demonstrated that Defb2 fused to the lymphoma antigen sFv also possesses chemoattractant activity for dendritic cells. This thesis also analyses the chemoattractant activity of Defb2 and Defr1 for neutrophils, dendritic cells and $CD4^+$ T-cells.

Synthetic Defb2 and Defr1 peptides were used in the studies introduced above. However, such peptides are expensive and doubts persist as to their correct folding and N-terminal sequence. Therefore, this thesis also describes an attempt to establish a cell culture-based system for the production of recombinant β-defensin peptides.

Finally, novel human β-defensins have been identified by bioinformatics techniques and another aim of this thesis is to investigate the expression of these genes by RT-PCR in a variety of human tissues.

2. Materials and Methods

2.1 Materials

Restriction enzymes, Proteinase K, RNase A, DNase 1, Polynucleotide kinase, T4 DNA ligase, T4 DNA polymerase, Shrimp alkaline phosphatase and first-strand cDNA synthesis kits were obtained from Roche, Lewes, East Sussex. Taq polymerase from Perkin Elmer Applied Biosystems, Roche Molecular Systems Inc, New Jersey, USA. Gel isolation, PCR purification and Nucleotide-removal kits were obtained from Qiagen, UK. DNA size markers λ/*Hind* III came from Roche and the 1 kb ladder was from GibcoBRL; standard and low melting point (LMP) agarose was from Sigma, Sigma-Aldrich company Ltd, Dorset; high grade NuSieve agarose was purchased from Biowhitaker. The membrane used for DNA and RNA blotting was from React Scientific, Ayrshire, UK. Dialysis tubing for size exclusion and removal of salt from tissue culture media was purchased form Fisher Scientific, UK. RNAzol used in isolation of RNA was purchased from Biogensis Ltd, Poole, Dorset. Diethylpyrocarbonate (DEPC) for dH_2O treatment came from Sigma. Chloroform, phenol and isopropanol were certified as DNAse- and RNase- free and were purchased from Sigma. Foetal calf serum (FCS), media and supplements for cell culture were obtained from GibcoBRL. Tissue culture flasks were supplied by Nunc Intermed, Roskilde, Denmark and Corning Costar, High Wycombe, Buckinghamshire. The control chemokines used in the cell migration studies were purchased from R&D Systems, Abingdon, UK. The filters used in the chemotaxis studies were from Osmonics. The 48-well microchemotaxis chamber was form Neuroprobe. Harris haemotoxylin and xylene were purchased from Fisher

Scientific UK Ltd, Loughborough, UK All mice used were C57Bl/6J and were supplied from Charles River UK Ltd. All mice were female and aged between 6 to 12 weeks. All radioisotopes came from Amersham International PLC, Amersham, Buckinghamshire. Oligonucleotide probes hybridisation and DNA primers for PCR were purchased form MWG Biotech.Bacto-agar and L-Broth for general bacterial culture were purchased from DIFCO laboratories, Detroit, USA. Agar and broth for culture of specific bacterial strains used in antibacterial experiments was purchased from Oxoid. Plasmids pOPRSV/MCSI and pBluescript II SK, were obtained from Stratagene Ltd, Cambridge. pGEN-II was from Promega. All other chemicals and reagents were of molecular biology grade and were obtained from Sigma, Sigma-Aldrich Company Ltd, Poole, BDH Laboratory supplies, Poole or Pharmacia Biotech AB, Uppsala, Sweden.

2.2 Standard Molecular Biology Techniques

2.2.1 Restriction Enzyme Digestion of DNA

Plasmid DNA was digested with 5-10 units enzyme/μg DNA for 1 hr. Genomic DNA was digested overnight with 10 units enzyme/μg DNA. Digestions were then analysed by gel electrophoresis.

2.2.2 Agarose Gel Electrophoresis of DNA

Agarose gel electrophoresis of DNA was performed using standard gel-running apparatus with agarose concentrations between 0.8-2.0 % w/v depending on the desired fragment resolution. Gels consisting of 3 % NuSieve agarose gel were used for resolution of DNA fragments below 200

base pairs (bp). For electrophoresis of DNA for use as a radioactive-labelled probe the digested DNA was run on a 1.5% low melting point agarose gel. Agarose was dissolved and cast in TBE buffer (0.09 M Tris-borate; 0.002 M EDTA (pH 8.0), which was also used as the running buffer. Ethidium bromide (10 μg/100ml) was added directly to the molten agarose. Gels were photographed under UV illumination.

2.2.3 Agarose Gel Electrophoresis of RNA

10-20 μg of total RNA were resuspended in a total volume of 20 μl sample buffer (50 % formamide, 2.2 M formaldehyde, 1 x MOPS (20 mM MOPS; 5 mM sodium acetate; 1 mM EDTA)) and incubated at 55°C for 10 min then placed on ice. 5 μl of 6 x formaldehyde gel-loading buffer (50 % glycerol, 1 mM EDTA (pH 8.0), 0.25 % bromophenol blue, 0.25 % xylene cyanol FF) were added to each sample and then loaded immediately onto the formaldehyde gel (1 % agarose; 1 x MOPS; and 18 % formaldehyde). An RNA sample containing 0.1 μl of ethidium bromide (10 mg/ml) was also loaded. The gel was run in 1 x MOPS at 50-80 V for 4-8 hr with recirculating buffer.

2.2.4 Purification of DNA Fragments from Agarose Gels

Using UV illumination the fragment of interest was excised with the minimum amount of agarose. Purification was carried out using QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions and eluting the purified DNA in 30 μl dH_2O. For isolation of DNA from LMP

agarose the band was excised, incubated with 3 volumes of dH_2O (v/w) and heated at 65°C for 3 min or until the gel slice had completely dissolved.

2.2.5 Analysis of Protein Content of β-Defensin Transfected Cells

The protein content of β-defensin transfected C127 cell lines were analysed by electrophoresis of culture media, cell lysates and purified media/lysates on a tris-tricine gel according to the method of Schagger and von Jagow (1987). This system uses a two-layer resolving gel and is appropriate for the separation of low molecular weight peptides in the MiniProtean II system (Biorad, herts, UK). The gel apparatus were set up according to the manufacturer's instructions. The lower resolving gel, composed of 16.5% acrylamide (30% stock solution of 29:1 acyrlamide:bisacrylamide (Severn Biotech Ltd, Worcs, UK) and 10% glycerol in 1x Gel Buffer (3M Tris, 0.3% SDS, pH8.45), was polymerised by the addition of 100 µl of a 10% ammonium persulfate (AMPS) solution and 10 µl TEMED. Simultaneous with this, the upper resolving gel (or spacer gel)of 10% acrylamide in 0.5% gel buffer was polymerised and immediately layered over the lower resolving gel. When this had set, the stacking gel (4% acrylamide in 0.25% gel buffer) was, polymerised by the addition of 50 µl AMPS and 10 µl TEMED and layered on top of the resolving gel.

The gel was placed inside the electrophoresis tank and the lower (anode) buffer (0.2 M Tris) and upper (cathode) buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) were added to the tank. 30 ml of culture media (cell lysates or purified media/lysate) were added to an equal volume of loading buffer (4% SDS, 12% glycerol, 50 mM Tris, 2 mM 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8), bolied for 5 min and returned immediately to ice.

The protein samples were loaded on the gel with low molecular weight markers (Sigma) and the gel was run for 2 hrs at 110 V. Following electrophoresis, the gel was washed three times in dH_2O, fixed in a solution of 50% methanol, 10% acetic acid for 30 min and stained with Bio-safe Coomassie G-250 (Biorad) according to the manufacturers instructions to reveal protein content of the loaded samples.

2.3 Preparation of DNA

2.3.1 Preparation of Genomic DNA from Cell Lines

Cell pellets were incubated overnight at 50°C in 1 ml of Quick Lysis Buffer (100 mM Tris HCl pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) containing 50 μg of Proteinase K. 1 ml of phenol (pH6.8) was added to the digest, mixed and then spun at 3 000 rpm for 5 min. The top layer was transferred to a fresh tube and an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol was added. The tube was mixed and centrifuged as before. The top layer was transferred to a new tube and equal volume of chloroform was added, and again the contents mixed and centrifuged as before. The top layer was transferred to a new tube, to which 2.5 volumes of 100% ethanol and 0.1 volumes of 3 M ammonium acetate was added. The tube was vortexed and spun as before and the precipitated DNA spooled onto glass rods. The DNA was washed with 70 % ethanol and then air-dried for 5 minutes, after which it was resuspended in 50 - 100 μl of TE (10 mM Tris HCl, 0.1 mM EDTA, pH 4.0).

2.3.2 Rapid Small-Scale Plasmid Preparation for Restriction Enzyme Analysis

5 ml of L-Broth, containing 50 μg/ml ampicillin, was inoculated with a single bacterial colony using a sterile pipette tip. The cultures were incubated overnight at 37°C. 1.5 ml of the cell cultures was centrifuged and the supernatant resuspended in 100 μl of STET buffer (8 % sucrose; 0.5 % Triton-X100; 50 mM EDTA (pH 8.0); 10 mM Tris-Cl (pH 8.0)). 100 μl of lysozyme (2 mg/ml) in STET buffer were added to each tube, mixed and left at room temperature for 5 min. The samples were then boiled for 1 min and centrifuged at 15 000 rpm for 10 min. The resulting pellet was removed with a toothpick and the DNA precipiated with 3 μl 3 M ammonium acetate and 250 μl isopropanol, mixed and centrifuged at 15000 rpm for 10 min. The pellet was washed with 70 % ethanol, dried and resuspended in 50 μl TE (pH 8.0). RNase A (100 μg/ml) was included at the time of restriction enzyme analysis.

2.3.3 Small-Scale Preparation of High Quality Plasmid DNA for Sequencing

High quality DNA suitable for sequencing was prepared using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. The DNA was eluted in 30 μl of dH_2O and the concentration assessed by spectrophotometry.

2.3.4 Large-Scale Preparation of High Quality DNA

A 5 ml starter culture was prepared overnight. This was then added to 500 ml of L-Broth containing ampicillin at 100 μg/ml and incubated for 16 hr at 37°C. The culture was then centrifuged in 2 x 250 ml Sorvall bottles at 8 000 rpm for 5 min at 4°C. Both pellets were resuspended in a total of 20 ml of GET solution (0.05 M glucose; 0.025 M Tris, 0.01 M EDTA (pH 8.0)) and a small amount of lysozyme added and left on ice for 5 min. 40 ml of freshly prepared 0.2 M sodium hydroxide/1 % SDS was added, mixed by inversion and incubated on ice for 10 min. Then 30 ml of potassium acetate buffer (60 ml 5M potassium acetate; 11.5 ml glacial acetic acid and 28.5 ml dH_2O) were added, mixed by inversion and incubated on ice for 10 min. The samples were then centrifuged at 12000 rpm for 20 min and the resulting supernatant decanted to a fresh bottle by straining through muslin. The DNA was then precipitated by the addition of 0.6 volumes of isopropanol, mixed and centrifuged at 12000 rpm for 10 min. The pellet was washed with 70 % ethanol, dried and resuspended in 2.5 ml TE (pH 8.0) before being transferred to a universal containing 2.75 g caesium chloride. 1 mg of ethidium bromide was added and the solution transferred to an ultracentrifuge tube. The tubes were heat sealed and centrifuged at 80000 rpm overnight. The DNA, which was present as an ethidium bromide stained band, was removed using a 21 G needle, transferred to a universal tube and the ethidium bromide washed out with H_2O-saturated butanol. The DNA was then precipitated by the addition of 10 ml ethanol and centrifuged at 5000 rpm for 15 min. The pellet was washed in 70 % ethanol, dried and then resuspended in the desired volume of TE.

2.4 Preparation of RNA

2.4.1 Preparation of RNA from Cells and Tissues

3 ml RNAzol B was added to confluent cells in a 75 cm^2 tissue culture flask, and the flask incubated at room temperature for 3 minutes. When the cells were completely lysed they were transferred to a fresh tube containing 300 µl chloroform and vortexed for 15 sec followed by a 5 min incubation on ice. The samples were then centrifuged at 15000 rpm for 15 min at 4°C and the top layer transferred to a fresh tube on ice. The RNA was precipitated by the addition of 4 µl glycogen and 500 µl of isopropanol and incubation at 4°C for at least 15 min. The sample was then centrifuged at 15000 rpm for 15 min and the resulting RNA pellet washed with 70 % ethanol and dried. The RNA was resuspended in 35 µl DEPC treated H_2O and the quantity and quality of the preparation assessed by spectrophotometry. RNA was isolated from whole tissues following the same method except the sample was homogenized using an Omni portable homogeniser (Camlab limited).

2.4.2 DNase Treatment of RNA

RNA samples were treated with DNase1 to remove any DNA present. The RNA samples were treated in a total volume of 50 µl containing 5 µl of 10 x DNase1 buffer, 10 units of DNase 1 (RNase free) and 40 units of RNase inhibitor. The samples were incubated at 37°C for 60 min and then extracted using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) by vortex and then centrifugation for 15 min at 15000 rpm. The top layer was transferred to a fresh tube and an equal volume of chloroform was added. The samples were then vortexed and centrifuged for 15 min at 15000 rpm.

The top layer was then transferred to a fresh tube on ice and the sample precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.6) and 2 volumes of ethanol and incubated at -70°C for 30 min. The sample was then centrifuged at 15 000 rpm for 15 min and the resulting pellet washed with 70 % ethanol. The pellet was dried and resuspended in the desired volume of DEPC treated H_2O and stored at -70°C.

2.5 General Cloning Techniques

2.5.1 Removal of 5' Phosphate Groups from Plasmid DNA

DNA was dephosphorylated with shrimp intestinal alkaline-phosphatase (SIP) to minimise the recircularisation of plasmid during ligation reactions. 10-20 μg of plasmid DNA were digested as described earlier, the reaction was then heated at 65°C for 10 mins to inactivate the restriction enzyme(s). The tube was placed on ice and 1/10 volume of SIP buffer and 1 ml of SIP enzyme was added, and the tube was incubated at room temperature for 15 mins. The DNA extracted with an equal volume phenol:chloroform:isoamyl alcohol. The DNA was then precipitated with 2.5 volumes of ethanol for 20 min at 4°C and recovered by centrifugation at 12 000 rpm for 30 min. The DNA was then resuspended in 90 μl TE buffer (pH 8.0).

2.5.2 Ligation of Fragments

Fragments with sticky complementary ends were ligated overnight at 15°C, using 1 unit of T4 DNA ligase (Roche). A molar ratio of 3:1 between insert and linearised vector was used with the concentration of vector at

approximately 50 ng. Commercially supplied 10 x ligation buffer was used and the volume of the reaction kept to a minimum, usually 10 µl.

2.6 Bacterial Transformations

2.6.1 Bacterial Transformation - Electroporation

Electrocomp™ TOP10F' cells (Invitrogen), were used for electroporation and a Bio-Rad Gene Pulser™ and Bio-Rad Pulse Controller were used for the electroporation procedure with capacitance set at 25 mFD, volts at 2.5 kV and resistance at 200 Ohms. 2 µl of ligation reaction were added to 40 µl of cells and incubated on ice for 1 min. The sample was then transferred to an electroporation cuvette, placed in the electroporation chamber and the electric charge discharged. 450 µl of room temperature L-Broth were added immediately after electroporation and the sample transferred to a 15 ml polypropylene tube. Samples were incubated with shaking at 37°C for 60 min. 10-100 µl of sample were spread onto LB plates (1.5 % w/v bacto-agar in L-broth) supplemented with the appropriate antibiotic. The plates were incubated overnight at 37°C.

2.6.2 Bacterial Transformation - Heat Shock Transformation

TOP10F' One Shot™ Competent Cells (Invitrogen) or DH5α library effeciency cells (Invitogen) were used for heat shock transformation. 3 µl of ligation reaction were added to 40 µl of cells and incubated on ice for 30 min. The sample was heat shocked at 42°C for 30 sec, placed on ice for 2 min and then 250 µl of L-Broth added. The tubes were shaken at 37°C for 1 hr and then spread onto plates as above.

2.7 Direct Cloning of PCR Products

PCR products were cloned into vectors in one of two ways. PCR products generated by Taq polymerase have 3′ deoxyadenosine overhangs and use of vectors containing single 3′ deoxythymidine residues allows PCR inserts to ligate efficiently with the vector. Direct PCR cloning was achieved using the pTOPO-2.1 vector. Briefly, 1 μl of fresh PCR product (less then 24 hr old) was incubated with 1 μl of TOPO vector and 1 μl of buffer and incubated for 30 minutes at room temperature. The reaction mix was then placed on ice and bacteria transformed as described in section 2.6.

Alternatively, the pGEM-T® vector was used. 3 μl of PCR product was mixed with 1 μl of pGEM-T vector, 5μl of rapid ligation buffer and 1 μl of T4 DNA ligase. The reaction was mixed and incubated for 1 hr at room temperature; this was followed immediately by transformation as in section 2.6.2.

2.8 Amplification of DNA and RNA by the Polymerase Chain Reaction (PCR).

All PCR reactions were performed using plugged RNase- and DNase-free tips.

2.8.1 Amplification from DNA

DNA was amplified according to Saiki *et al.* (1988). A 50 µl reaction containing up to 1 µg of genomic DNA or 1 ng of plasmid DNA, 5 µl 10 x PCR buffer (P), 5 µl of 25 mM $MgCl_2$, 1 µl of 50 x dNTP mix (10 mM of each nucleotide) and 100 nm of each primer was prepared in a 0.5 ml thin-walled PCR tube (Advanced Biotechnologies) or 96-well thermoplate. 2.5 units of thermostable DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) were added to the mixture. Thermal cycling was controlled by a programmable heating block (OmniGene, Hybaid). As the defensin genes are all of a similar size a highly similar PCR amplification conditions could be used for all reactions, the annealing temperature (Ta) was adjusted, depending on the melting point (Tm) of the amplimers used, but generally the annealing temperature was at least 5°C below the melting temperature.

Step 1: 94°C for 3 mins,

35 cycles of:

Step 2: 94°C for 1 min,

Step 3: Ta°C for 30 secs,

Step 4: 72°C for 1 min,

Step 5: 72°C for 10 mins.

Gene	Amplimer Name	Amplimer Sequence	Ta(°C)
DEFB1	*DEFB1* F1 (5')	TCCAAAGGAGCCAGCCTCTC	55
	DEFB1 R1 (3')	AAAAAGTTCATTTCACTTCTGCGTC	55
DEFB2	*DEFB4* F1 (5')	CCAGCCATCAGCCATGAGGGT	56
	DEFB4 R1 (3')	GGAGCCCTTTCTGAATCCGCA	56
DEFB105	*DEFB105* F1 (5')	CGTGCTCTTCTTTCTGACCC	56
	DEFB105 R1 (3')	GTTCTTCATTTTTCCCGCAA	56
DEFB106	*DEFB106* F1 (5')	TCTATTTGCTATGTTCTTCATTTTGG	56
	DEFB106 R1 (3')	GCAGCAGAGAAAGTTCAGCC	56
DEFB107	*DEFB107* F2 (5')	TGAGACTCCATTTGCTTCTCC	56
	DEFB107 R2 (3')	GCAGGCACCAATTTGATCTT	56
DEFB108	*DEFB108* F1 (5')	TGCTGTCCTCTTCTTCACCA	56
	DEFB108 R1 (3')	CGGCTATTTAAACATCTCCCA	56
DEFB109	*DEFB109* F1 (5')	TTTTGGCTGCTCTCATTCTTC	56
	DEFB109 R1 (3')	TGCAGCAAAATGGTGCTAAT	56
h***GAPDH***	*hGAPDH* F1 (5')	GGGAGCCAAAAGGGTCATC	55
	hGAPDH R1 (3')	CACCGTCACTACCGTACCTG	55
Defb1	*Defb1* F1 (5')	CACTCTGGACCCTGGCTGCC	54
	Defb1 R1 (3')	AATCCATCGCTCGTCCTTTA	54
Defb2	*Defb2* F1 (5')	GCCATGAGGACTCTCTGCTC	54
	Defb2 R1 (3')	TGTCACTTGACTTCCATGGC	54
mβ *-Actin*	β-*Actin* F1 (5')	GGCCCAGAGCAAGAGAGGTATCC	55
	β *-Actin* R2 (3')	ACGCACGATTTCCCTCTCAGC	55
Sequencing from plasmids	*T3*	AATTAACCCTCACTAAAGGG	50
	T7	GTAATACGACTCACTATAGGGC	50
	Ks	TCGAGGTCGACGGTATC	50
	Sp6	GATTTAGGTGACACTATAG	50

Table 2.1 Sequence of Amplimers used in PCR and RT-PCR

2.8.2 Amplification from RNA

First-strand cDNA synthesis was accomplished using a First-strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche). Briefly, duplicate samples of 2 μg of total RNA in a total volume of 7.8 μl in DEPC treated H_2O were denatured at 65°C for 15 min. To one set of samples were added 2 μl 10 x PCR buffer, 4 μl $MgCl_2$, 2 μl 10 mM dNTPs, 0.4 μl gelatin, 2 μl of random primer p(dN)6, 40 units RNase Inhibitor, 0.8 μl AMV reverse transcriptase. The other was set up identically except that AMV reverse transcriptase was omitted and replaced with DEPC-treated dH_2O to verify for RNA amplification. A minus RNA control was also included to assay for contamination. The samples were incubated at room temperature for 10 min to allow the primers to anneal and then placed at 42°C for 1 hr followed by 5min at 95°C to inactivate the enzyme. After the tubes had been cooled on ice, 5 μl of each reaction were used in a standard PCR reaction as described in section 2.8.1.

2.9 Transfer of RNA and DNA

2.9.1 Southern Blot Transfer of DNA

The DNA fragments were separated by agarose gel, and then the gel was soaked in denature buffer (0.5 M sodium hydroxide; 1.5 M sodium chloride) for 20 min before being briefly rinsed and then soaked in neutralisation buffer (0.5 M Tris; 1.5 M sodium chloride) for 20 min. The gel was then transferred onto a nitrocellulose membrane by capillary transfer overnight in 20 x SSC (3 M sodium chloride; 0.3 M sodium citrate). The membrane was fixed by baking at 80°C for 20 min and then UV stratalinked (Stratalinker

1800, Stratagene). The membrane was then hybridised as described in section 2.10.1 or 2.10.2.

2.9.2 Northern Blot Transfer of RNA

RNA was separated by electrophoresis and the lane containing the RNA-ethidium bromide was excised and photographed under UV light to visualise separation. The gel washed 3 x 20 min in dH_2O and then blotted onto a nitrocellulose membrane as described in section 2.9.1 but using DEPC-treated reagents. Hybridisation was performed as described in section 2.10.1.

2.9.3 Construction of Bacterial Colony Hybridisation Filters

A nitrocellulose filter (0.45 μm pore) was placed on a LB agar plate and marked for orientation. Individual bacterial colonies were then streaked within a gridded box on the filter and the plate was incubated overnight at 37°C. The filters were removed from the plate and floated in 10 % SDS (colony side up) for 1 min to lyse the cells and left to dry on filter paper for 1 min. The filters were submerged in denaturing solution (0.5 M sodium hydroxide; 1.5 M sodium chloride) for 5 min and then neutralisation buffer (0.5 M Tris; 1.5 M sodium chloride) for 5 min followed by 2 x SSC for 30 sec and left to dry on filter paper for 30 min. The DNA was fixed onto the membrane by baking at 80°C.

2.10 Radioactive Hybridisation

2.10.1 Preparation of Radioactively-Labelled DNA Probes

Approximately 60 ng of purified DNA in a total volume of 12 µl were boiled for 10 min to ensure denaturation and then labelled using 4 µl of High Prime (Roche) and 30 µCi [α-32P] dCTP and the reaction was incubated at 37°C for 30 min. Incorporation was assessed by purifying the probe through a NIK column (Amersham International PLC, UK). The probe was stripped by boiling for 10 min with 1 mg sonicated salmon sperm and then added to the prehybridised filters.

2.10.2 Preparation of Radioactively-Labelled Oligonucleotide Probes

Labelled probes from oligonucleotides 15-25 bp in length were made by end-labelling. Approximately 50 ng of oligonucleotide probe were labelled in a total volume of 20 µl containing 2 µl of 10 x polynucleotide kinase (PNK) buffer, 10 units of PNK and 30 µCi [γ–32P] dATP. The reaction was incubated at 37°C for 1 hr and then added directly to the prehybridised filters.

2.10.3 Hybridisation of Radioactive DNA Probes.

Filters were pre-hybridised at 68°C for 2 hr in rotating hybridisation bottles with 20-30 ml hybridisation solution (6 M sodium chloride, 0.6 M trisodium citrate, 0.4% sodium dodecyl sulfate, 0.2% sodium pyrophosphate, 2x

Denhardts (50x Denhardts, 1% polyvinyl pyrrolidine, 1% bovine serum albumin, 1% Ficoll 400) and 1% of 10 mg/ml of sonicated salmon sperm). After pre-hybridisation, the radiolabelled probe was added directly to the solution and the blots hybridised overnight at 68°C. Filters were then washed with hybridisation wash (3 M sodium chloride, 0.3 M trisodium citrate, 0.1% sodium dodecyl sulfate) for 2 x 20 min. The background radiation was monitored with a Geiger counter and if a high-level of background radiation persisted the washes were repeated but with the SSC concentration reduced to 1 x and, if required, 0.1 x SSC. The filters were exposed to Kodak X-OMAT AR film at -70°C in cassettes containing intensifying screens phosphor-imager intensifying screen at room temperature.

2.10.4 Hybridisation of Radioactive Oligonucleotide Probes

Filters were pre-hybridised at 48°C for a minimum of 4 hr in rotating hybridisation bottles with 20-30 ml oligonucleotide hybridisation buffer (3 M sodium chloride, 0.3 M trisodium citrate, 0.4% sodium dodecyl sulfate and 0.2% sodium pyrophosphate) and 1 mg sonicated salmon sperm. After pre-hybridisation, radiolabelled oligonucleotide probe (Table 2.2) was added directly to the solution and the blots hybridised for 4 hr at a temperature of 4-6°C lower than the oligo Tm. Filters were then washed with 6 M sodium chloride, 0.6 M trisodium citrate and 0.1% sodium dodecyl sulfate. The filters were then exposed to Kodak X-OMAT AR film at -70°C in cassettes containing intensifying screens or a phosphor-imager intensifying screen at room temperature.

2.11 DNA Sequencing

Sequencing of double strand DNA was performed using the ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). 500 ng of double stranded DNA were amplified in a total volume of 20 µl containing 18 ng or 3.2 pmol of amplimer (Table 2.1) Terminator Ready Reaction Mix in 0.2 ml Micro-Tubes (Advanced Biotechnologies Ltd) with the following cycling conditions: 25 cycles of 96°C for 30 sec; 50°C for 15 sec; 60°C for 4 min. The resulting products were then precipitated by the addition of 2 µl 3 M sodium acetate and 50 µl 95 % ethanol and centrifuged at 15000 rpm for 30 min. The resulting pellet was washed with 70 % ethanol and dried in a speedivac. Samples were electrophoresed on a ABI 377 sequencing machine (Perkin Elmer Applied Biosystems) and the results analysed using Sequencing analysis software ImageQuant™ (Molecular Dynamics).

Gene	Internal Oligonucleotide Probe
DEFB1	GGTGGTAACTTTCTCACAGG
DEFB4	CCAGTCTTTTGCCCTAG
DEFB105	TACAGGGAAGGTGATCGGA G
DEFB106	TTCAACTGCCATCAGGTGAG
DEFB107	CCAGTAAGAGGTGGTTTGGG
DEFB108	GCCAAGTTCTACCAGCCAAG
DEFB109	TCACTGTGAAGCCGAATGTC
Defb1	AATGCCTTCAACATGGAGG
Defb2	TCAGAGCCATTTGTCCTCCT

Table 2.2: Sequence of Oligonucleotide Probes.
Sequence reads 5' to 3'.

2.12 Cell Isolation and Culture

2.12.1 Culture of C127 and HBE cell lines

The C127 mouse mammary epithelial cell line and human bronchial epithelial (HBE) cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 5-10% foetal calf serum (FCS) L-glutamine, 500 Units penicillin and 50 mg/ml streptomycin, and the cells incubated at 37°C in humidified incubator with 5% CO_2. When approximately 90% confluent, the cells were subcultured into a fresh culture flask. The monolayer was washed once with versene (0.48% EDTA in Dulbeccos's PBS)) and then incubated at 37°C with 3 ml of trypsin solution (0.075% in Hanks Buffered Saline Solution, Sigma), preheated to 37°C for 5 min or until all the cells had detached from the surface. 12 ml of culture media was added to stop the reaction and 1-5 ml of the cell suspension was transferred to a new culture vessel.

2.12.2 Transfection of C127 and HBE Cell Lines

The C127 and HBE cell lines were transfected by the calcium phosphate method. One day prior to the transfection cells were trypsinised from the culture vessel and resuspended in a 6 mm-diameter culture dish at a density of 5x 10^6 cells/dish, giving approximately 50-70% confluence after 24 hrs. 10 μg of linearised defensin-pOPRSVII/MCS or empty vector was resuspended in 218.75 μl of 0.1% TE buffer (1 mM Tris, 0.1 mM EDTA, pH8.0), and transferred to a sterile 5 ml Falcon tube. To this 31.25 μl of 2 M Calcium Phosphate, with gentle agitation (generated by gently blowing air bubbles through the mixture) 250 μl of 2 x HEPES-buffered saline (HBS, 5 M sodium

chloride and 1 m HEPES) was added over a 30 second period. The complex of calcium phosphate precipitation was allowed to form over 20 minutes with constant agitation. Media was removed from the cells to be transfected and the monolayer washed twice with phosphate buffered saline (PBS) (Sigma, UK). The calcium-phosphate suspension was added directly to the monolayer and the cells were incubated at 37°C for 15 min. After this period, 4 ml of complete media was added and the cells incubated for a further 4 hours. The media-precipitate mixture was removed and the monolayer washed once in media.

The monolayer was then treated with a solution of 24% DMSO in HBS for 3 min at room temperature; the monolayer were then washed three-times with serum-free media, complete media was added and the cells incubated for 24 hrs as normal. After this time the cells were subcultured, as described above, 1:3 into 6-well plates and G418-selection was added to the culture media at a concentration of 500 mg/ml and the cells were incubated as normal.

2.12.3 Selection of C127 Defensin-Transfected Clones

Transfected cells were grown in presence of the selection for approximately 14-21 days, after this time most cells had died and single G418-resistant colonies could be seen. When sufficiently large the clones were trypsinised from the culture flask as described earlier and cultured in a 48-well culture dish with continued selection. The procedure was repeated to allow the selected clones to be cultured in successively larger vessels up to a 75 cm^2 culture flask.

2.12.4 Harvesting of Cell Lysates and Culture Media

Following approximately 48 hr of a confluent cell monolayer maintained in 25 ml of media (as described above with 5% FCS), the media was harvested into a 50 ml Falcon tube and frozen at –70°C. The cell monolayer was harvested by trypsinisation, as described earlier. The cell pellets were resuspended in dH_2O and lysed with a few strokes of a homogenizer and the lysate was then stored at -70°C.

2.12.5 Dialysis of Cell Lysates and Culture Media

Dialysis was performed in dialysis tubing with a molecular weight cut-off of 500 Da (10 mm diameter). A 15-20 cm length of tubing was cut and rinsed briefly in PBS and one end was closed with a clamp, 10ml of media was pipetted into the tubing, which was then sealed at the open-end with another clamp. The tubing was the submerged completely into a minimum of 5 litres of dH_2O and left to dialyse overnight. The clamp at one end was removed and the dialysed media was drained into a sterile vessel and frozen at -70°C until required.

2.12.6 Purification of cell lysates and culture media

Media and cell pellets were purified using a SepPak C_{18} carboxymethyl cartridge (Waters) according to the method of Diamond *et al.* (1991). 40 ml of media or 5 cell pellets (from T75cm^2 culture flasks) were retrieved from –70°C storage and cells were resuspended in 40 ml PBS and homogenised with a few strokes of a Omni portable homogeniser. Trifluoroacetic acid (TFA) was added to the media to a final volume of 0.1%. The cartridges were

conditioned with 10 ml of a 60:40 solution of acetic acid:0.1% TFA and then washed with 10 ml of 0.1% TFA. The sample was then loaded onto the column at a flow rate of 5-10 ml/min. The column was washed with 10 ml of 0.1% TFA and then eluted with 4 ml of 60:40 acetic acid:0.1% TFA. The elutant was then freeze-dried for 24 overnight and resuspended in 1 ml of phosphate buffer (see section 2.14.2).

2.12.7 Isolation of Neutrophils

Mice were injected intraperitoneally with 0.5 ml 9% casein solution. 24 hr later the mice were injected with a second dose of casein and 3 hr later killed by cervical dislocation. The peritoneal cavity was lavaged with 5 ml DMEM/0.5mM EDTA and the collected fluid placed on ice. 16 ml ice cold 0.83 % ammonium chloride (pH 7.4) were added to the lavage fluid tolyse contaminating red blood cells. The suspension was incubated at room temperature for 15 min then 30 ml 1 x Hanks Buffered Salt Solution (HBSS) were added and the sample centrifuged for 10 min at 1450 rpm. The supernatant was discarded and 10 ml HBSS added to the cells and centrifuged at 775 rpm for 10 min after which the supernatant was discarded and the cells resuspended in 5 ml HBSS and centrifuged at 775 rpm for 5 min. The resulting pellet was resuspended in 500 µl PBS. The total cell count was performed using a Neubauer haemocytometer chamber and the percentage of neutrophils estimated from a differentially stained cytospin (Cytospin, Shandon Scientific). Samples of at least 90 % neutrophils were used for the chemotaxis assay and the cells were resuspended in DMEM/0.5 mM EDTA at a concentration of 5×10^6 cells/ml.

2.12.8 Isolation of CD4+ T-lymphocytes

CD4+ T-cells were isolated from mouse spleens using positive selection protocol in an autoMACS magnetic cell-sorter (Miltenyi Biotec). The mice were killed by cervical dislocation and the spleen removed and placed into RPMI media supplemented with 10% FCS, L-glutamine, 500 units penicillin and 50 mg/ml streptomycin. The spleen was disassociated by passage through a 100 μm cell filter with the rubber end of the plunger from a 1 ml syringe into 30 ml of media as above to give a single-cell suspension. The cells were spun at 300 x g at 4°C for 7 min. The pellet was resuspended in 1 ml of PBS Dulbecco's PBS without calcium and magnesium (Sigma, UK) and 4 ml of red blood cell (RBC) lysis buffer (1 mM ammonium carbonate and 114 mM ammonium chloride) and incubated on ice for 5 mins. Immediately this time was up 10 ml of Dulbecco's PBS was added to stop any further lysis and the cells spun as before. The pellet was resuspended in 10 ml of PBS and the cells counted in a haemocytometer. The cells were resuspended in MACs buffer (PBS + 0.5% BSA) at a concentration of $1x10^7$ cells/90 μl (≈1.11×10^8 cells/ml), this was supplemented with 10 μl of MACS CD4+ (L3T4) MicroBeads (Miltenyi Biotec) per 1×10^7 cells. The cell:MicroBead suspension was incubated at 4°C for 15 mins; 30 ml of MACS buffer was added to stop further reaction and the cells were spun as before for 10 mins. The cells were resuspended at a concentration of 2×10^8 cells/ml and run on the positive selection programme POSSEL1 on the autoMACS. The positive fraction was eluted in 2 ml of MACS buffer, the volume was adjusted to 10 ml and the cells counted. The cells were centrifuged as before and resuspended in chemotactic media (RPMI 1640 +10% foetal calf serum) at 5×10^6 cells/ml. The cells were incubated at 37°C prior to the chemotaxis experiment. The purity

of the cell population was assessed the analysing the proportion of cells positive for CD3 and CD4 by fluorescence-activated cell sorting (FACS).

2.12.9 Culture of Bone Marrow-Derived Dendritic Cells.

2.12.9.1 Isolation of Bone Marrow Cells and Basic Culturing

Bone marrow-derived dendritic cells were cultured from bone marrow cells isolated from mouse tibia and femur bones following the method of Inaba *et al* (1992). The mice were killed by cervical dislocation and the long bones of the hind legs were removed. The bones were cleaned of muscle and place into complete media (as detailed in section 2.12.8). The bones were broken at the knee and cleaned further, both ends of the bones were cut with a scalpel to create an open tube. The 2 ml syringe was filled with media and using a 23G needle the marrow was washed out of the bones into fresh media. Clumps of marrow were broken up into single cells by passage through the 25G needle. The cells were centrifuged at 300 x g for 7 min at 4°C. The resultant pellet was resuspended in 1 ml PBS, 4 ml of RBC lysis buffer was added and the suspension incubated on ice for 5 min. At the end of this period the suspension was centrifuged as before, the cells washed 3 times with 10 ml of complete media and resuspended in 20 ml of complete media. The cells were counted in a haemocytometer and the volume adjusted to give 3.75 x 10^5 cells/ml in complete media supplemented with 5% (500 U/ml) of supernatant from the granulocyte-macrophage colony stimulating factor (GM-CSF) -expressing cell line (Stoiber *et al.*, 1995) and the cells cultured in a 24-well plate with 1 ml per well.

2.12.9.2 Preparation of Day 6 Immature Dendritic Cells

The GM-CSF supplemented culture media was replenished on day 3 of the experiment (with day 0 being the start). The plate was swirled gently but firmly 20 times to aid the removal of non-adherent cells, (mainly contaminating granulocytes). The media was removed from half the wells using a 1 ml pastette, fresh medium was gently added and the process repeated with the remaining wells. The cells are sufficiently developed for use on day 6. The "loosely-adherent" day 6 immature dendritic cells (d6iDC) are harvested by swirling the plate as described above and then vigorously pipetting around the well with a 1 ml pastette 6-10 times. The cells are centrifuged as above and washed twice in chemotactic media. The cells were counted in a haemocytometer and the concentration adjusted to 5×10^6 cells/ml. The cells were the incubated at 37°C for at about 1 hr prior to usage in the chemotaxis experiments.

2.12.9.3 Preparation of Day 7 Immature Dendritic Cells

7 day-old immature dendritic cells (d7iDC) were cultured as in section 2.12.9.2, except that on day 6 the media was replenished as on day 3 and the cells harvested on day 7.

2.12.9.4 Preparation of Mature Dendritic Cells

For the preparation of mature dendritic cells the above protocol (2.12.9.3) was followed but 100 ng/ml of *Escherichia coli* LPS was added to the culture media on day 7 to mature the dendritic cells.

2.12.10 Fluorescence Associated Cell Sorting (FACS) Analysis

Identification of the cell population under investigation was conducted by fluorescent analysis for expression cell surface markers using a FACS can flow cytometer (Coulter Corporation, USA). All antibodies used were purchased form Pharmingen/Becton Dickinson (USA). The labelling antibodies used, and the corresponding isotype, used as specificity control are detailed in Table 2.3.

MACs-isolated splenic CD4+T-lymphocytes were double stained for the CD4 and CD3 cell surface markers. Dendritic cell populations were analysed by double staining for CD11c and MHC-II expression, and single-stained for CD54 and CD86 expression. The level of contamination was assessed by single staining for the granulocyte marker Gr1 and the B-cell marker CD45.

Marker	Labelling Antibody	Isotypic Antibody
CD4	FITC anti-mouse CD4 (L3T4)	FITC rat $IgG_{2b}\kappa$
CD3	PE anti-mouse CD3e	PE Hamster $IgG_1\lambda$ (anti-TNP)
CD11c	PE anti-mouse CD11c (HL3)	PE Hamster $IgG_1\lambda$ (anti-TNP)
MHC-II	FITC anti-mouse $I\text{-}A^b$ ($A_B{}^b$)	FITC Mouse $IgG_{2a}\kappa$ (anti-TNP)
CD54	PE anti-mouse CD54 (ICAM-1)	PE Hamster $IgG_1\kappa$ (anti-TNP)
CD86	PE anti-mouse CD86 (B7-2)	PE Rat $IgG_{2b}\kappa$
Gr1	PE anti-mouse Ly-6G (Gr1)	PE rat $IgG_{2b}\kappa$
CD45	FITC anti-mouse CD45/B220	PE rat $IgG_{2a}\kappa$

Table 2.3: Cell surface markers used to identify the population of cell under study. Antibodies were conjugated either to phycoerythrin (PE) or fluorescein isothiocyanate (FITC).

For direct antibody labelling 5 x 10^5 cells/sample were added to a flexible 96 round-bottom well plate and centrifuged at 300 x g for 3 min at room temperature. The antibodies were diluted to a final concentration of 5 μg/ml in 100 μl of FACS buffer (PBS + 0.5% BSA, 10% mouse serum, 0.1% sodium azide). The appropriate antibody or antibodies were added to the corresponding well and the cells incubated in the dark on ice for 30 min. The cells were washed twice in FACS buffer, and finally resuspended in 400 μl of 1:1 FACs buffer/FACS fix (Dulbecco's PBS + 4% paraformaldehyde). Cell sorting by flow cytometry was carried out within 5 days after staining.

2.13 Culture of Bacteria for Analysis of Defensin Antibacterial Function

Bacteria were provided by Professor John Govan and prepared by Wendy Hannant, Dr Cathy Doherty, or Wendy Hannant, Department of Medical Microbiology, University of Edinburgh. The bacterial strains used were; *Staphylococcus aureus* CF clinical isolate C1705, *Pseudomonas aeruginosa* CF clinical isolate J1385 and J1532 and strain PAO1, *Escherichia coli* clinical isolates J2408 and J3201, and *Burkholderia cenocepacia* CF clinical isolate J2315. *Pseudomonas aeruginosa* CF clinical isolate J1385 has been documented to have preferentially infected several CF individuals, despite exposure to other strains simultaneously in a jacuzzi, and its mucinophilic strain is called J1532 (Nelson *et al* 1990). *Burkholderia cenocepacia* CF clinical isolate J2315, is the virulent Edinburgh-Toronto epidemic strain ET/12 (Govan and Deretic 1996).

An aliquot of the appropriate strain was recovered from storage, in 10% w/v skimmed milk at –70 °C, thawed and streaked on the appropriate media.

This was incubated at 37 °C overnight (for 48 hr for *B. cenocepacia*). Nutrient broth (25 g/L, 0.5% w/v Yeast extract; Oxoid Ltd., Hampshire, UK) was inoculated with a few colonies and incubated at 37 °C overnight in an orbital incubator. The bacterial suspension was centrifuged at 3000x g for 10 minutes and the supernatant removed. The bacterial pellet was resuspended in 0.85% saline and standardised using a spectrophotometer at OD_{590}, to a density previously determined to correspond to ~ 1×10^9 CFU/ml.

The following media were used: *S. aureus* was grown on Blood agar (39 g/L Columbia base agar (Oxoid Ltd., Basingstoke, Hampshire, UK), 5% defibrinated horse blood (E & O Laboratories Ltd., Burnhouse, Bonnybridge, Scotland) added after autoclaving and cooling), *P. aeruginosa* was grown on Pseudomonas isolation agar (45 g/L, 2% glycerol; Difco Laboratories Ltd., West Molesey, Surrey, UK), *Escherichia coli* was grown on MacConkey agar (44.5 g/L; Mast Diagnostics, Mast Group Ltd., Bootle, Merseyside, UK), *B. cenocepacia* was grown on Cepacia medium (32.5 g/L, Ticarcillin 100mg/L, Polymixin B 300,000 u/L; Mast Diagnostics, Mast Group Ltd., Merseyside, UK).

2.14 Analysis of Defensin function

2.14.1 Synthesis of peptides

Synthetic defensin peptides (DEFB2, Defb2 and Defr1) were designed to represent the mature portion of the prepropeptides of DEFB2, Defb2 and Defr1 respectively. The peptides were purchased from and synthesised by Albachem Ltd., King's Buildings, Edinburgh, UK, as described below.

2.14.2 Antibacterial Assays

The optimisation of this method was performed by Drs Donald Davidson and Duncan Borthwick, MRC Human Genetics Unit, Edinburgh, UK.

Fresh, overnight cultures of bacteria were prepared as described in section 2.3 and supplied in 0.85% saline at ~ 1 x 10^9 colony froming units per ml. Immediately prior to use, 1 ml aliquots of bacterial suspension were centrifuged at 1000x g for 10 minutes at room temperature and resuspended in 10 ml of 10 mM Phosphate buffer (8 mM K_2HPO_4, 2 mM KH_2PO_4, 5.6 mM D-Glucose) containing either 0 mM, 30 mM, 60 mM, 90 mM, 120 mM or 150 mM NaCl (buffer pH 7.60 at 0mM NaCl, pH 7.40 at 30 mM NaCl, pH 7.33 at 60 mM NaCl, pH 7.27 at 90 mM NaCl, pH 7.23 at 120 mM NaCl and pH 7.20 at 150 mM NaCl).

A 1 in 50 dilution of the bacterial suspension was performed in the same buffer providing a suspension of ~ 2 x 10^6 CFU per ml. Two sets of duplicate 500 µl reactions were prepared containing 50 µl of either 10 mM Phosphate buffer alone or 10x stock of synthetic peptide (0.5 mg/ml to give a final concentration of 0.05 mg/ml unless otherwise stated) rehydrated in 10 mM Phosphate buffer, 47.5 µl of 10 x stock of NaCl in 10mM Phosphate buffer (0 mM, 300 mM, 600 mM, 900 mM, 1.2 M or 1.5 M NaCl) and 377.5 µl of 10mM Phosphate buffer, to which 25 µl of bacterial suspension (~ 5 x 10^4 CFU) in the appropriate salt concentration was added.

These reactions were incubated for 30 minutes at 37 °C, after which duplicate sets of serial, enfold dilutions were prepared from each sample, in 10 mM Phosphate buffer with the appropriate concentration of NaCl. 100 µl samples were plated out on the appropriate agar, incubated at 37 °C for 24 hours (48 hours for *B. cenocepacia*), colony counts were performed and extrapolated to provide the CFU count for the neat sample. The anti-microbial activity of the peptide was compared against buffer alone, to control for the effects on the bacteria of varying the NaCl environment. These studies were repeated to ensure the validity of results, reproducing the salt-sensitive antibacterial profile on a minimum of two separate occasions for each set of conditions.

The assessment of the antibacterial activity of defensin-transfected cells lines were conducted by following the same method except that phosphate buffer, salt and peptide solution, to which the bacteria were added was replaced by 475 µl of media, lysate or purified lysate and media solution resuspended in phosphate buffer.

2.14.3 Statistical Analysis

The data were classified by factors of Salt concentration; treated or untreated with peptide and the number of 'replicate sources. It was found that a $Log_e(1+x)$ transformation of the counts was best for stabilising the between-group variances. The data were transformed and averaged over the repeated counts. A three-factor balanced analysis of variance was done using the three-factor interaction as the error term. When other two-factor terms were found to be non significant, they were removed from the model and the

fitted values recalculated. This increases the number of degrees of freedom for error and reduces the confidence range.

The 'human and 'mouse' data were analysed separately. Tables of the transformed data were produced with standard errors, and the data were transformed back to the original scale to give estimates of counts and 95% confidence limits classified by Salt concentration treatment and 'replicate' . The response of the different 'replicates' was sufficiently different that they could not be averaged.

The differences between the corresponding transformed values for Untreated and Treated were calculated (Peptide – Blank) . The same form of analysis of variance was done and fitted values calculated. By transforming the means back to the linear scale it was possible to estimate the likely proportion of cells reduced by peptide under the varying salt levels – or '% Kill' and also give 95% confidence limits. Negative '% Kill' values reflect the possibility of cell counts actually being higher under high salt concentrations.

2.14.4 Cell Migration Assays

30 μl of β-defensin at 0, 0.1, 1, 10, 100, 1000, 10000 ng/ml in chemotactic media or the control chemoattractant, which had been preheated to 37°C and vortexed, were added to the bottom chamber of a Neuroprobe Standard 48 Well Chemotaxis Chamber (Neuro Probe, Inc). All samples were assayed in triplicate. A Nuclepore Polycarbonate membrane (Costar) was placed on top of the bottom chamber followed by the silicon gasket and then the top chamber to which 50 μl of the neutrophil suspension was added to each well.

The chamber was incubated in a humid 5 % CO_2 incubator at 37°C for 1 hr for neutrophils, 1.5 hr for T-cells and 4.5 hr for dendritic cells after which the chamber was inverted and the filter removed (migrated cells side up). The side of the filter with non-migrating neutrophils was washed in PBS and scraped across a blade to remove the cells.

2.14.5 Staining of Filters

For neutrophils the filter was then fixed and stained using Diff-Quik® (Dade®), placed on a 76 mm x 52 mm microscope slide and the number of neutrophils migrated in each well counted. For all other cell types, the membrane was fixed in methanol and then stained in Harris' haematoxylin (0.4%) for 4 min, then washed in water for 1 min. The filter was briefly dried and then placed in Scotts' tap water (0.2 M sodium bicarbonate and 0.8 M magnesium sulfate) for 2 min and again rinsed in water. The membrane was dehydrated by incubation for 1 min in increasing concentrations of industrial methylated spirits (64% > 74% > 100%), and then submerged in xylene for 1 min before being mounted on a slide. Membranes were viewed by brightfield microscopy at 400 x magnification and three random of fields of view (FOV) were counted per well.

2.14.6 Statistical Analysis

The counts in each well were classified by Experiment, Agent and Concentration. Counts were made from three aspects A, B and C. The data were transformed to log(x) (or log(x)+1 if the data contained a zero) and ((x+3/8)) respectively. Means were taken of the transformed and

untransformed data classified by the factors Experiment, Agent and Concentration and tested for homogeneity of variance between each of the treatment groups classified by the factors Agent and Concentration.

A preliminary analysis of variance was done with the model including all main effects and two-factor interactions of the factors Experiment, Agent and Concentration. When either of the two-factor interactions with Experiment were not significant ($p > 0.2$) they were removed form the model. This helped to increase the degrees of freedom available for estimating error.

Analysis of Variance was then repeated for each case with the (possibly) modified model, and produced a table classified by the factors Agent x Concentration, which contained means, standard errors of means and Minimum Significant (at 5%) Difference (MSD).

Chapter 3: The Antibacterial Activity of β-Defensins.

3.1 Introduction

Antimicrobial peptides comprise an important aspect of the innate immune system; their main function is to kill invading microorganisms. There exists many different families of antibacterial peptides; one of the largest, found in plants, insects and vertebrates is the defensin family. In vertebrates, defensins are classified into three subfamilies which are called α-, β- or θ-defensins depending on the connectivity of the intramolecular disulfide bridges. Whilst the activity of α-defensins has been fairly well documented, the function of β-defensins has been less well characterised. However, these peptides came to wider acknowledgement when their dysfunction was implicated in the pathogenesis of cystic fibrosis (CF) lung disease (Goldman *et al.*, 1997).

A defect in the antibacterial activity of the airway surface liquid (ASL) of the lungs was implicated in the lung pathogenesis of CF. The ASL of primary cultures of human airway epithelial cells has been demonstrated to possess salt-sensitive antibacterial activity that was inhibited by the addition of NaCl. In contrast, cultures of airway epithelia from CF individuals showed markedly reduced antibacterial activity, and this could be restored by reducing the concentration of salt in the ASL (Smith *et al.*, 1996). Further work suggested that the expression of CFTR was required for maximal absorption of NaCl across the epithelium, and thus, CF cultures show reduced absorption of salt, and elevated levels of NaCl in the ASL (Zabner *et al.*, 1998). Together, these data suggest that the loss of functional CFTR in CF

elevates NaCl concentrations, which reduces the antibacterial activity of the ASL and that this may predispose CF airways to bacterial infections. However, other theories explaining CF pathogenesis have been proposed (see section 1.6) and due to the difficulty in measuring the salt concentration of ASL the 'defensin/elevated-salt' theory and the role of defensins in CF pathogenesis remain controversial. Indeed, a recent study found evidence for the existence of a salt-independent defect in the antibacterial activity of CF ASL and suggested that absence or dysfunction of an as yet unidentified antimicrobial factor(s) may explain the reduced antimicrobial activity (Bals *et al.*, 2001).

The ASL contains a multiplicity of antimicrobial factors such as defensins, cathelicidins, secretory leukocyte protease inhibitor (SLPI), lysozyme and lactoferrin. Studies carried out using a human bronchial xenograft model system suggested that DEFB1 (hBD1) may play a central role in the antibacterial activity that is compromised in CF (Goldman *et al.*, 1997). Ablation of the expression of *DEFB1* using anti-sense RNA directed against the 5′ region of *DEFB1* cDNA was shown to abrogate almost completely the antibacterial activity of the ASL, and furthermore synthetic DEFB1 peptide was shown to possess salt-sensitive antibacterial activity.

These experiments broadened the interest in the activity of β-defensins. Investigation of the salt-sensitive nature of the antibacterial activity of DEFB1 and a murine homologue Defb1 was conducted by Dr Donald Davidson, working in this lab (Morrison *et al.*, 1998; Morrison *et al.*, 2002b). These experiments revealed that synthetic both DEFB1 and Defb1 possessed significant salt-sensitive antimicrobial activity at a concentration of 50 μg/ml. This activity was broad range, killing *P. aeruginosa* CF isolate J1385, *S. aureus*

CF isolate C1705 and *E. coli* clinical isolate J2408. However, the spectrum of activities differed between the two peptides; DEFB1 showed the highest activity against *P. aeruginosa* J1385, and lowest against *E. coli* J2408, whereas Defb1 killed *S. aureus* C1705 most efficiently and showed least activity against *P. aeruginosa* J1385. These results may indicate the evolution of species differences in the specific activities of the different β-defensins against different bacteria. Further studies have been conducted mouse mutant models deficient in expression of a β-defensin. In one study Defb1 expression was disrupted by gene targeting (Morrison *et al.*, 2002a) however *S. aureus* were still efficiently cleared from the lungs these mice after nebuliseation. Although no overt deleterious pathogen was evident in these *Defb1*$^{-/-}$, there were significantly greater numbers of *S. aureus* bacteria in the urine of mutant mice compared to wildtype control. However, another mouse model with ablation of Defb1 expression has been reported (Moser *et al.*, 2002). Interestingly, this mouse model demonstrated delayed clearance of the CF pathogen *Haemophilus influenzae* compared to wildtype mice. Moreover, this study also reported that the cellular and cytokine responses in the mutant mice were normal, suggesting that the defective clearance was due to the loss of Defb1 antibacterial activity. Furthermore, a role for defensins in host defence is further suggested by an earlier study that prevented production of functional α-defensins (cryptidins) in the mouse intestine by ablating expression of matrilysin, an enzyme that processes the α-defensins to the function form (Wilson *et al.*, 1999). Bacteria administered to these Mat$^{-/-}$ mice survived in greater numbers and showed greater virulence than in wildtype mice. Thus, these data suggest that the cryptidins serve to protect the mouse intestinal surfaces from bacterial colonisation.

Since these initial studies were conducted many more β-defensins have been identified in both human and mice, but the precise function of β-defensins in the ASL of the lung and other tissues remains unknown. *DEFB4* (originally termed *hBD2* or *DEFB2*) was the second human β-defensin to be identified; it is expressed in the skin, lungs, uterus and trachea, but expression is induced in foreskin-derived fibroblasts in response to bacterial stimulus (Harder *et al.*, 1997) . Further studies have shown that synthetic a DEFB4 peptide possessed potent antimicrobial activity against Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*, and it also demonstrated synergistic activity with lysozyme and lactoferrin (Bals *et al.*, 1998b). An inducible β-defensin, *Defb2*, has also been identified in the mouse (Morrison *et al.*, 1999). Defb2 shows 36% similarity at the amino acid level to DEFB4 and 50% similarity to Defb1; it is expressed constitutively in the kidney and uterus, also expression can be stimulated in the trachea by bacterial LPS. The discovery of Defb2 raises interesting questions about its activity and salt-sensitivity as well as the degree of functional homology between murine and human β-defensins.

All of the defensins discussed above possess the characteristic six cysteines and have the typical pattern and spacing in their arrangement. Recently, however, a novel murine β-defensin has been identified that lacks the first canonical cysteine (Morrison *et al.*, 2002a). *Defensin-related 1* (*Defr1*) is expressed in the testis, uterus and heart, but expression was not induced in the trachea in response to LPS-treatment. At the amino acid level *Defr1* shows low (less than 50%) similarity to *Defb1* and *2* and has greater greatest similarity (73%) to Defb3.

The discovery of novel β-defensins also raises many important and interesting questions about the function of these antibacterial peptides and

their interaction with other defensins and also with the different components of the innate and adaptive immune systems. One of the most remarkable features of the different peptides is the pronounced sequence divergence in the mature peptide (Figure 3.1). This suggests that the evolution of β-defensins may have been driven by positive selection for amino acid substitutions (Hughes, 1999; Morrison *et al.*, 2002c). This would have lead to the rapid evolution of a large number of peptides with greatly divergent primary structures, and may have had the effect of increasing the spectrum of activity of β-defensins against potentially pathogenic microorganisms. The effect of this pronounced sequence divergence on the activity of the various β-defensins remains a largely unexplored area, and may have implications for the development of novel antibiotics.

```
Defb1 MKTHYFLLVMICFLFSQMEPGVGILTSLGRRTDQYKCLQHGGFCLRSSCPSNTKLQGTCK
Defb2 MRTLCSLLLICCLLFSYTTPAVGSLKSIGYEAELDHCHTNGGYCVRAICPPSARRPGSCF
DEFB1 MRTSYLLLFTLCLLLSEMASGGNFLTGLGHRSDHYNCVSSGGQCLYSACPIFTKIQGTCY
Defr1 MRIHYLLFT---FLLVLLSPLAAFSQKIN---DPVTYIRNGGICQYRCIGLRHK-IGTCG
DEFB4 MRVLYLLFS---FLFIFLMPLPGVFGGIG---DPVTCLKSGAICHPVFCPRRYKQIGTCG
      *     *      *                        ↑   *  *           * *

Defb1 PDKPNCCKS--
Defb2 PEKNPCCKYMK
DEFB1 RGKAKCCK---
Defr1 SP-FKCCK---
DEFB4 LPGTKCCKKP-
           ***
```

Figure 3.1: Alignment of Synthetic β-Defensin Peptides. Multiple sequence alignment of β-defensins whose functions have been analysed as synthetic peptides. Amino acids in red represent the sequence of the mature synthetic peptides tested, * indicates a direct match. Note that Defr1 lacks the first canonical cysteine (↑). Analysis was performed using the Clustal W program at www.hgmp.mrc.ac.uk with default settings.

To assess the antibacterial profile DEFB4, Defb2 and Defr1 these peptides were synthesised by Albachem Ltd (Edinburgh, UK) to the amino acid sequence of the proposed mature peptide (Figure 3.1). 5 x 10^4 colony-forming units (CFU) of bacteria were incubated for 30 min at 37°C with or

without the peptide in the presence of 0, 30, 90 or 150 mM NaCl. Duplicate reactions were performed and at the end of the incubation period, each reaction was itself plated out in duplicate on the appropriate agar (Figure 3.2). In order to assess the effect of salt alone on bacterial survival, control reactions were set up without peptide. The number of CFU in the control sample was compared to those from the peptide-treated samples at each NaCl concentration, and the quantity of bacteria surviving in the peptide sample was expressed as a percentage of the counts from the control sample (percent-kill). These studies will help to address several of the outstanding questions regarding the antibacterial activity and salt-sensitivity of these β-defensins. These experiments are designed to assess the activity of the synthetic peptides against a variety of CF and non-CF pathogens. They may also facilitate the understanding of the effect of different peptide sequences on the spectrum of antibacterial activity and the species differences between the activities of mouse and human defensins.

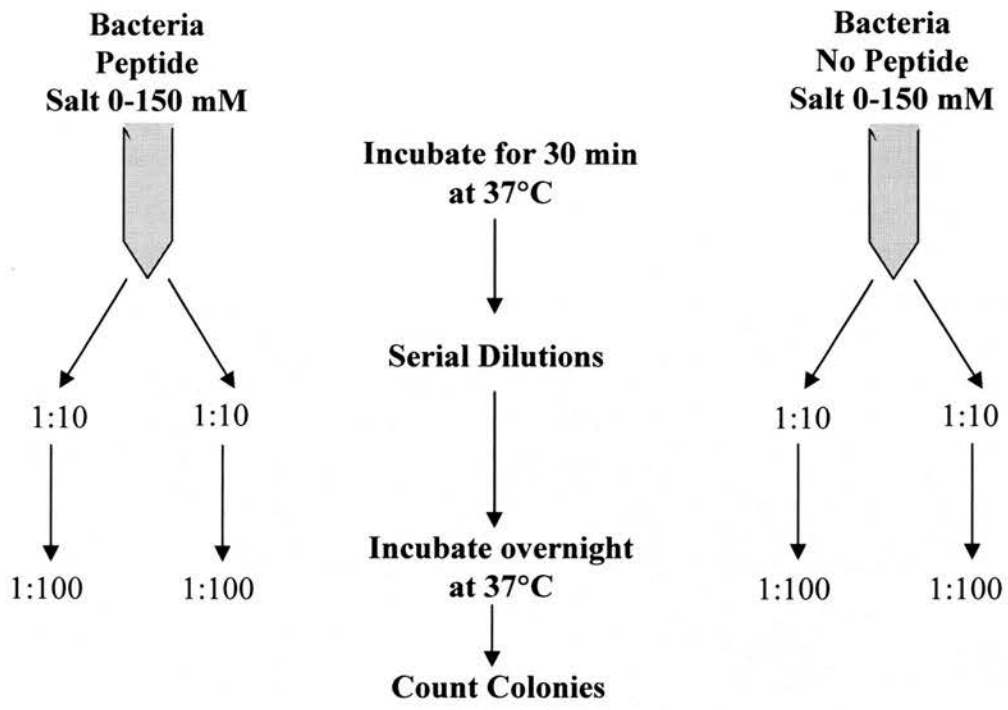

Figure 3.2: Schematic Diagram of Antibacterial Assays. Bacteria were incubated in phosphate buffer with or without the peptide at various salt concentrations. Following incubation at 37°C for 30 min, 1:10 and 1:100 serial dilutions of the bacterial suspension were made. These were plated out, incubated overnight and the numbers of surviving colony forming units (CFU) were counted. The reactions indicated above were set up in duplicate, and the whole experiment was repeated a minimum of three time (*i.e.* n=3).

3.2 Antibacterial Activity of DEFB4 and Defb2

The antibacterial activity of DEFB4 and Defb2 and their salt-sensitivity was assessed against a range of bacteria. These were the Gram-positive *Staphylococcus aureus* CF clinical isolate C1705, and the Gram-negatives *Pseudomonas aeruginosa* CF clinical isolates J1385 and J1532, and laboratory strain PAO1, *Burkholderia cenocepacia* CF clinical strain, epidemic strain J2315 and *Escherichia coli* (clinical isolates J2408 and J3201). The activity of DEFB4 against a range of pathogens has already been demonstrated (Harder *et al.*, 1997; Bals *et al.*, 1998b; Singh *et al.*, 1998), however its salt-sensitivity has not been fully characterised.

A previous study assessing the activity of DEFB1 against *P. aeruginosa* obtained similar bacterial killing with synthetic DEFB1 with a concentration ranging from 60 – 500 μg/ml (Goldman *et al.*, 1997). Moreover, work carried out in this laboratory on the activity of DEFB1 and Defb1 found significant antibacterial activity at a concentration of 50 μg/ml (Morrison *et al.*, 2002b) (Morrison *et al.*, 1998). For these reasons and to allow for easier comparison between the different studies a concentration of 50 μg/ml has also been used in the following experiments. Studies were tested for reproducibility in a minimum of three experiments (n=3) and representative experiments are illustrated below in Figures 3.3 –3.9.

3.2.1 Antibacterial activity of DEFB4 and Defb2 against *S. aureus* C1705.

At a concentration of 50 μg/ml and in the absence of NaCl, DEFB4 displayed high levels of activity and killed approximately 80% of bacteria. This fell to ≈40% in 30 and 90 mM NaCl, but killing was only 10% in 150 mM of NaCl (Figure 3.3 A and C). This antibacterial activity was statistically significant ($p < 0.01$) between 0 and 90 mM, but the low level of activity observed at 150 mM was not statistically significant. Defb2 was observed to exhibit lower levels of activity compared to DEFB4 at each NaCl concentration tested (Figure 3.3 B and D). Defb2 killed just over 40% of bacteria in the absence of NaCl, and this activity fell rapidly in the presence of NaCl. The percent-kill in 30 mM NaCl was 12% and the level of activity fell further in the 90 and 150 mM NaCl to just 5%. The activities seen in 0 mM and 30 mM NaCl were statistically significant ($p < 0.01$), however the very low levels observed in 90 and 150 mM NaCl were not significant. The activity of DEFB4 was significantly greater than that of Defb2 in the presence of 0 or 30 mM NaCl ($p < 0.01$) and also in 90 mM ($p < 0.05$).

The concentration of NaCl in the absence of peptide had no statistically significant affect on bacterial survival, with the level of survival in control sample remaining fairly constant across all NaCl concentrations tested. This suggests that the observed effects were due to the action of the peptides and their interaction with NaCl, and not due to the NaCl concentration alone.

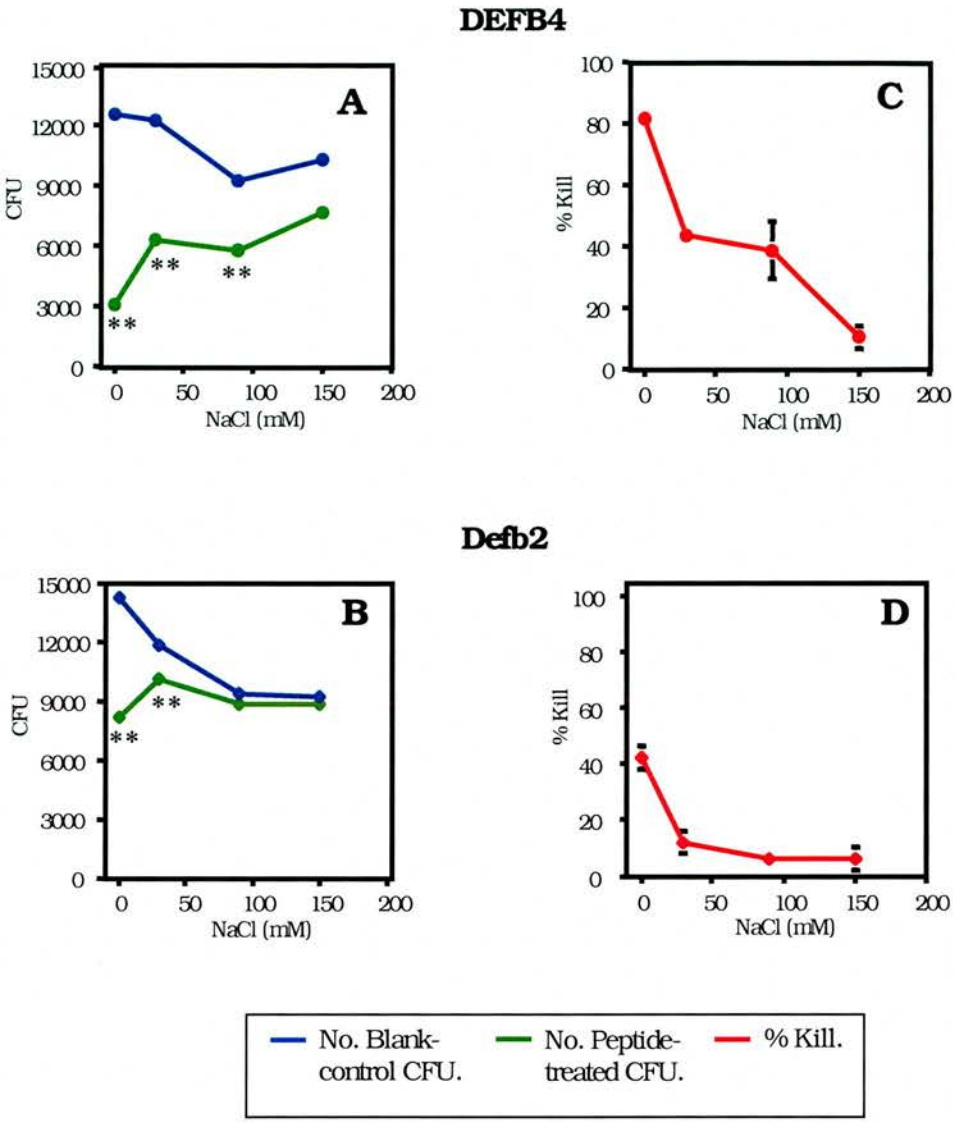

FIGURE 3.3: Antibacterial activity of DEFB4 and Defb2 against *Staphylococcus aureus* C1705.

Bacteria were incubated with the peptide at 50 μg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

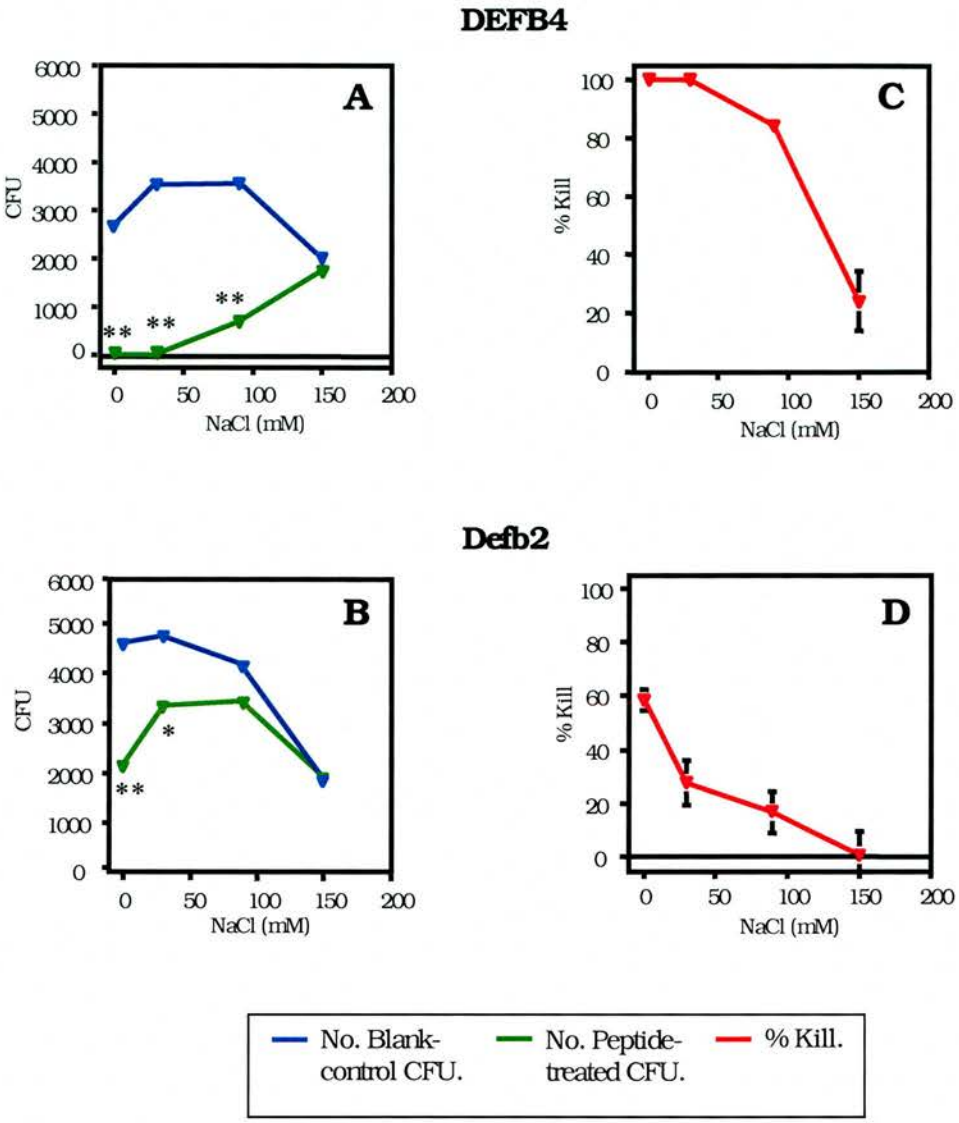

FIGURE 3.4: Antibacterial activity of DEFB4 and Defb2 against *Pseudomonas aeruginosa* J1385.

Bacteria were incubated with the peptide at 50 µg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

3.2.2 Antibacterial activity of DEFB4 and Defb2 against *P. aeruginosa* J1385.

DEFB4 also displayed statistically significant activity against *P. aeruginosa* J1385 (Figure 3.4 A and C). In the absence of salt and in 30 mM NaCl DEFB4 killed 100% of bacteria; this fell to 80% in the presence of 90 mM NaCl and killing was reduced again by 150 mM NaCl, with activity at approximately 20%. The activities seen in 0, 30 and 90 mM of NaCl were found to be statistically significant ($p < 0.01$) however, the activity in 150 mM NaCl was not significant. Again, Defb2 demonstrated considerably lower levels of activity compared to DEFB4 at each NaCl concentration examined (Figure 3.4 B and D). Killing by Defb2 was significantly lower at all concentrations ($p < 0.01$), except 150 mM, where no significant difference was observed. Killing of *P. aeruginosa* J1385 by Defb2 was 60% in the absence of NaCl, approximately 30% in the presence of 30 mM of NaCl. Both of these activities were significant, $p < 0.01$ in the absence of NaCl and $p < 0.05$ at 30 mM NaCl, however the reduced levels of activity observed at 90 and 150 mM were not statistically significant.

The level of NaCl alone did have a significant effect on the survival of *P. aeruginosa* J1385 in the control samples. This effect was greatest at the extremes of NaCl concentrations tested (0 and 150 mM) with significantly fewer bacteria surviving at these concentrations compared to the survival at 30 and 90 mM NaCl ($p < 0.01$). This pattern was also seen in the previous studies on the activity of DEFB1 and Defb1 against *P. aeruginoisa* J1385 (Donald Davidson, PhD Thesis, Edinburgh University, 2000). However, as these NaCl concentrations also represent the conditions under which the

greatest and least activities of DEFB4 and Defb2 are observed, this suggests that the effects of the peptides are independent from the effects of NaCl alone, although the low number of bacteria surviving in 150 mM did lead to quite variable results at this concentration of NaCl.

3.2.3 Antibacterial activity of DEFB4 and Defb2 against *P. aeruginosa* PAO1.

The activity of DEFB4 and Defb2 were also tested against the PAO1 laboratory strain of *P. aeruginosa*. DEFB4 demonstrated statistically significant killing ($p < 0.01$) at all NaCl concentrations tested (Figure 3.5 A-D). DEFB4 showed greater activity across all NaCl concentrations against *P. aeruginosa* PAO1 compared to *P. aeruginosa* J1385 (Figure 3.5 A and C). DEFB4 killed 100% of bacteria in 0 and 30 mM NaCl, this is the same level of activity observed against the *P. aeruginosa* J1385. However, the activity was greater against *P. aeruginosa* PAO1 than against *P. aeruginosa* J1385 at the higher salt concentrations, 95% and 80% killing compared to 85% and 25% in 90 mM and 150 mM NaCl respectively. These differences suggest that as with conventional antibiotics, the spectrum of antibacterial activities may be specific to different strains of bacteria.

Defb2 also killed *P. aeruginosa* PAO1 more efficiently at each NaCl concentration tested compared to *P. aeruginosa* J1385, and again, Defb2 was less effective than DEFB4 (Figure 3.5 B and D). The greatest activity of Defb2 against *P. aeruginosa* PAO1 was in the absence of salt at this level approximately 80% of

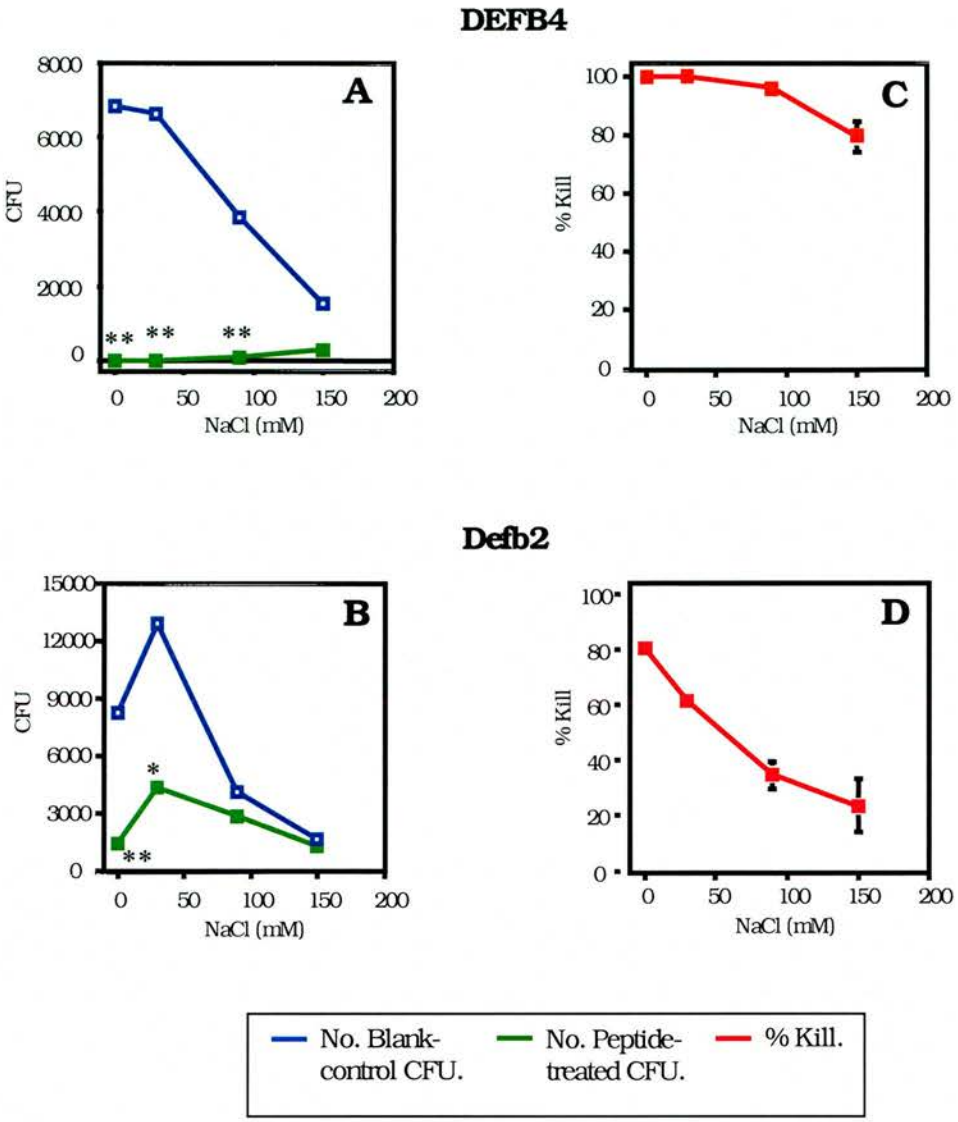

FIGURE 3.5: Antibacterial activity of DEFB4 and Defb2 against *Pseudomonas aeruginosa* PAO1.

Bacteria were incubated with the peptide at 50 µg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

bacteria were killed, this fell to 60% when the NaCl was elevated to 30 mM, and to ≈35% and ≈25% in 90 mM and 150 mM respectively. The effect of Defb2 was statistically significant compared to blank control at 0 mM ($p < 0.01$), and 30 and 90 mM NaCl ($p < 0.05$). However, the antibacterial activity of Defb2 was significantly lower than that of DEFB4 at all NaCl concentrations ($p < 0.01$ at 0-90 mM and $p < 0.05$ at 150 mM).

3.2.4 Antibacterial activity of DEFB4 and Defb2 against *P. aeruginosa* J1532.

The activity of DEFB4 and Defb2 was also examined against the *P. aeruginosa* J1532 (Figure 3.6 A to D). This is the mucoidy version of the CF isolate *P. aeruginosa* J1385. DEFB4 demonstrated very high, and statistically significant ($p < 0.01$), levels of antimicrobial activity against *P. aeruginosa* J1532 at all NaCl concentrations tested (Figure 3.6 A and C). At 0 mM and 30 mM DEFB4 killed 100% of bacteria; at higher concentrations of NaCl the activity was reduced to 95% (90 mM) and 85% (150mM). Defb2 also killed *P. aeruginosa* J1532 significantly more efficiently than it killed *P. aeruginosa* J1385 ($p < 0.01$). In the absence of NaCl Defb2 killed 75% of bacteria, the level of activity in the reduced compared to 0 mM but static between 30 mM and 150 mM NaCl, with 60% to 55% of bacteria killed and at 150 mM NaCl (Figure 3.6 B and D). The activities for both DEFB4 and Defb2 against *P. aeruginosa* J1532 were statistically significantly higher than their activities against the non-mucoid *P. aeruginosa* J1385

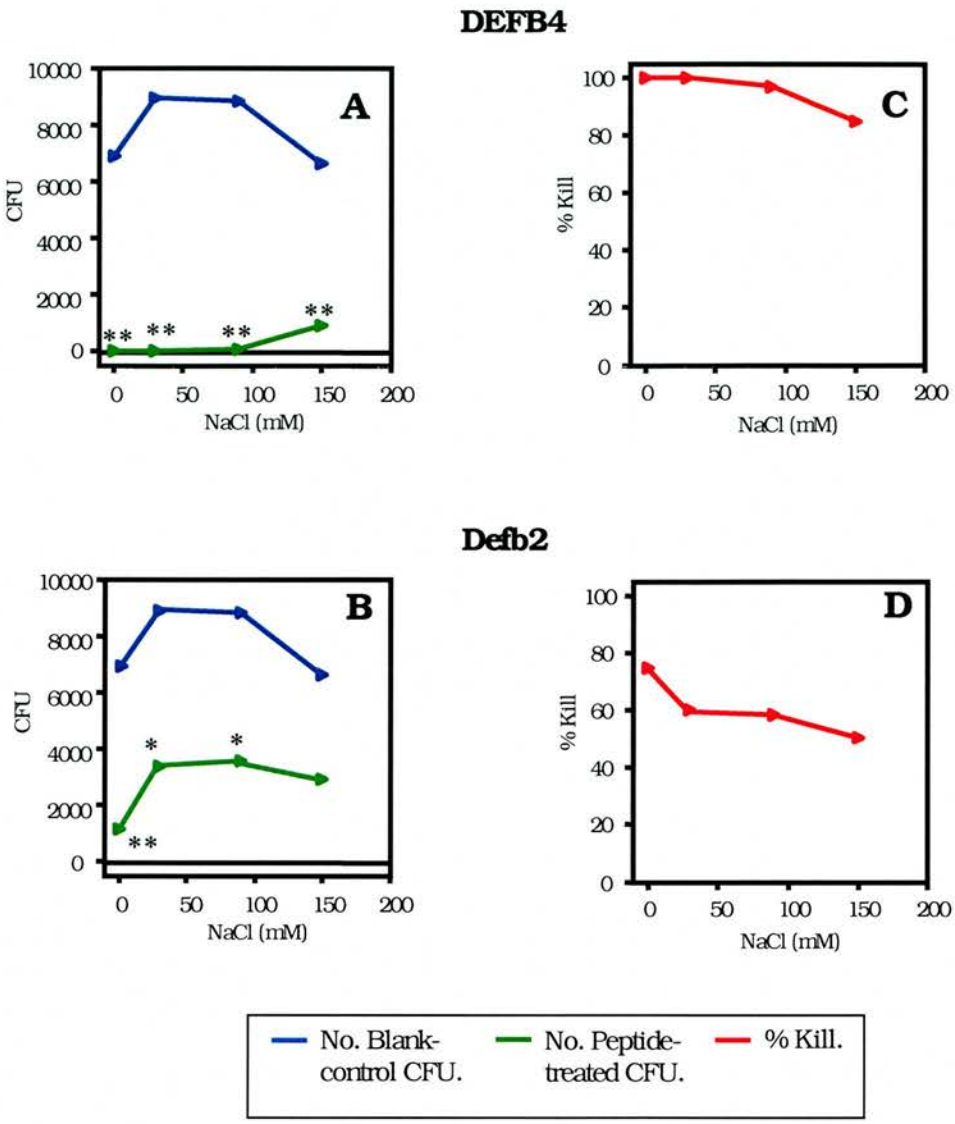

FIGURE 3.6: Antibacterial activity of DEFB4 and Defb2 against Mucoid *Pseudomonas aeruginosa* J1532.

Bacteria were incubated with the peptide at 50 µg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error of *S. aureus* C1705 from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

3.2.5 Antibacterial activity of DEFB4 and Defb2 against *E.coli* J2408 and J3201.

The antibacterial activities of DEFB4 and Defb2 against *E. coli* clinical isolate J2408 were also examined (Figure 3.7 A-D). Neither DEFB4 nor Defb2 demonstrated killing at any of the NaCl concentrations examined. However, weak activity was observed against another clinical strain, *E. coli* J3201. This level of activity was very similar for DEFB4 and Defb2, both killing at approximately 25% in the absence of NaCl and 15% at 30 mM NaCl ($p < 0.01$). Antibacterial activity was ablated by the presence of 90 or 150 mM NaCl (Figure 3.8 A to D).

3.2.6 Antibacterial activity of DEFB4 and Defb2 against *B. cenocepacia* J2315.

In agreement with previous β-defensin killing data, neither DEFB4 nor Defb2 had any effect on the survival of *B. cenocepacia* J2315, and no significant differences were observed between peptide and control sample at the 50 μg/ml at any of the NaCl concentrations tested (Figure 3.9 A to D). Previous work has found this bacterial strain to be resistant to the effects of defensins (Morrison *et al.*, 1998 and Hancock, 1997a).

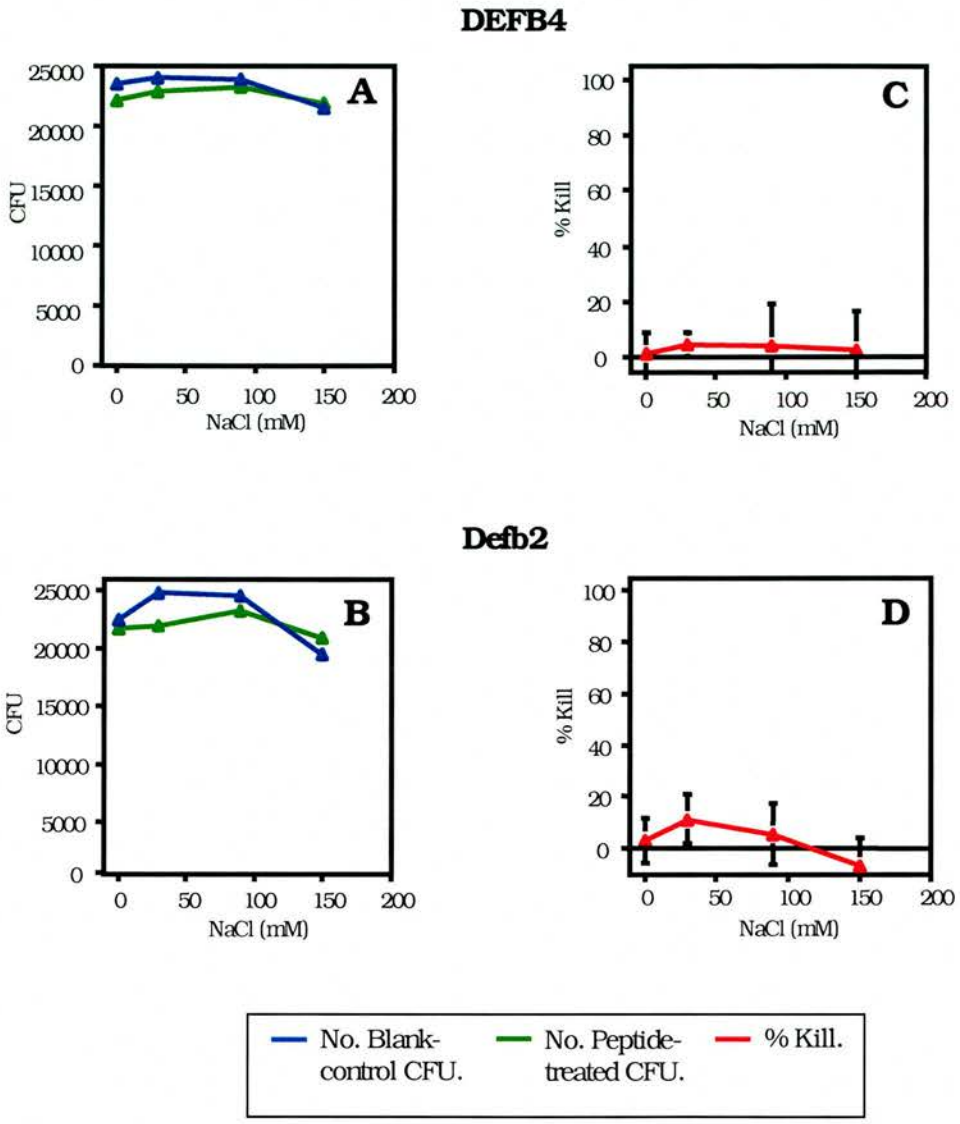

FIGURE 3.7: Antibacterial activity of DEFB4 and Defb2 against *Escherichia coli* J2408.

Bacteria were incubated with the peptide at 50 μg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

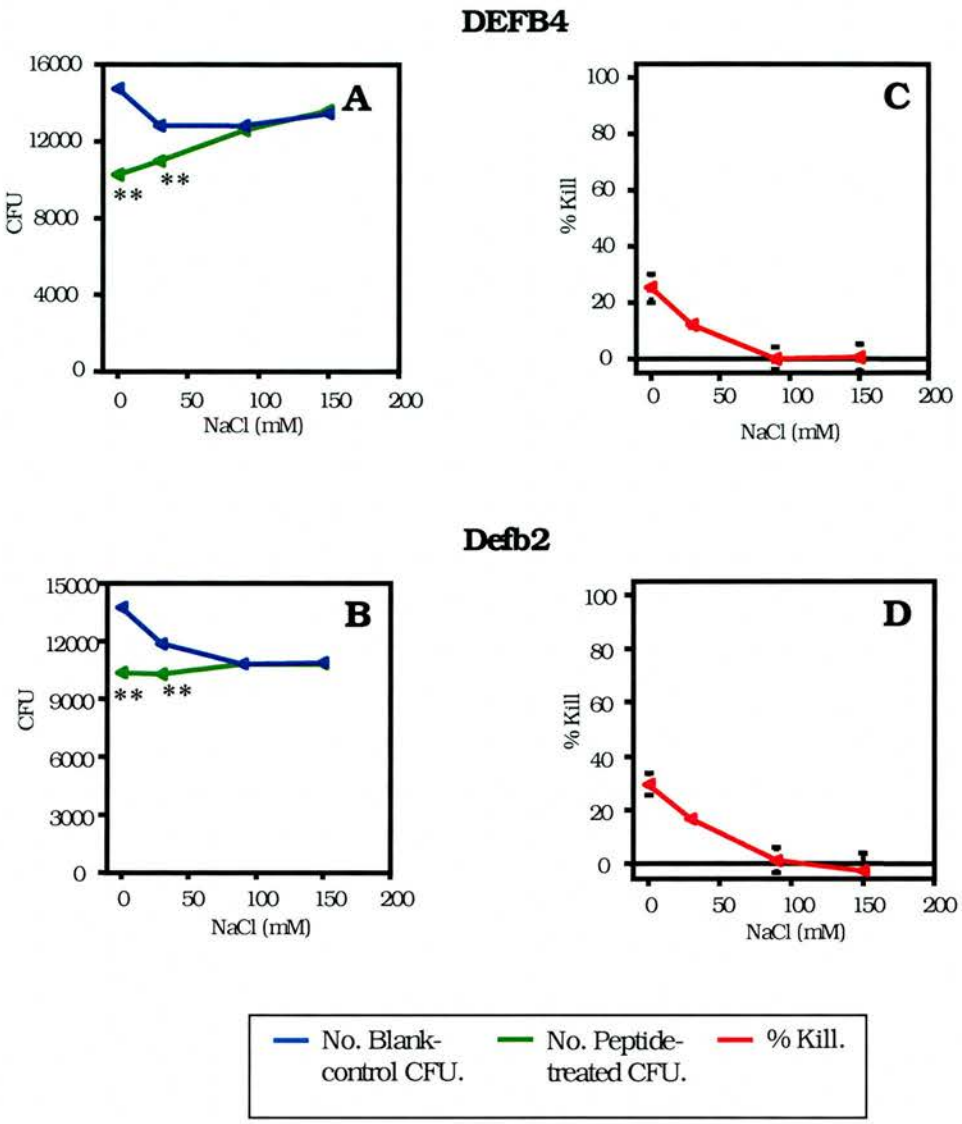

FIGURE 3.8: Antibacterial activity of DEFB4 and Defb2 against *Escherichia coli* J3201.

Bacteria were incubated with the peptide at 50 μg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

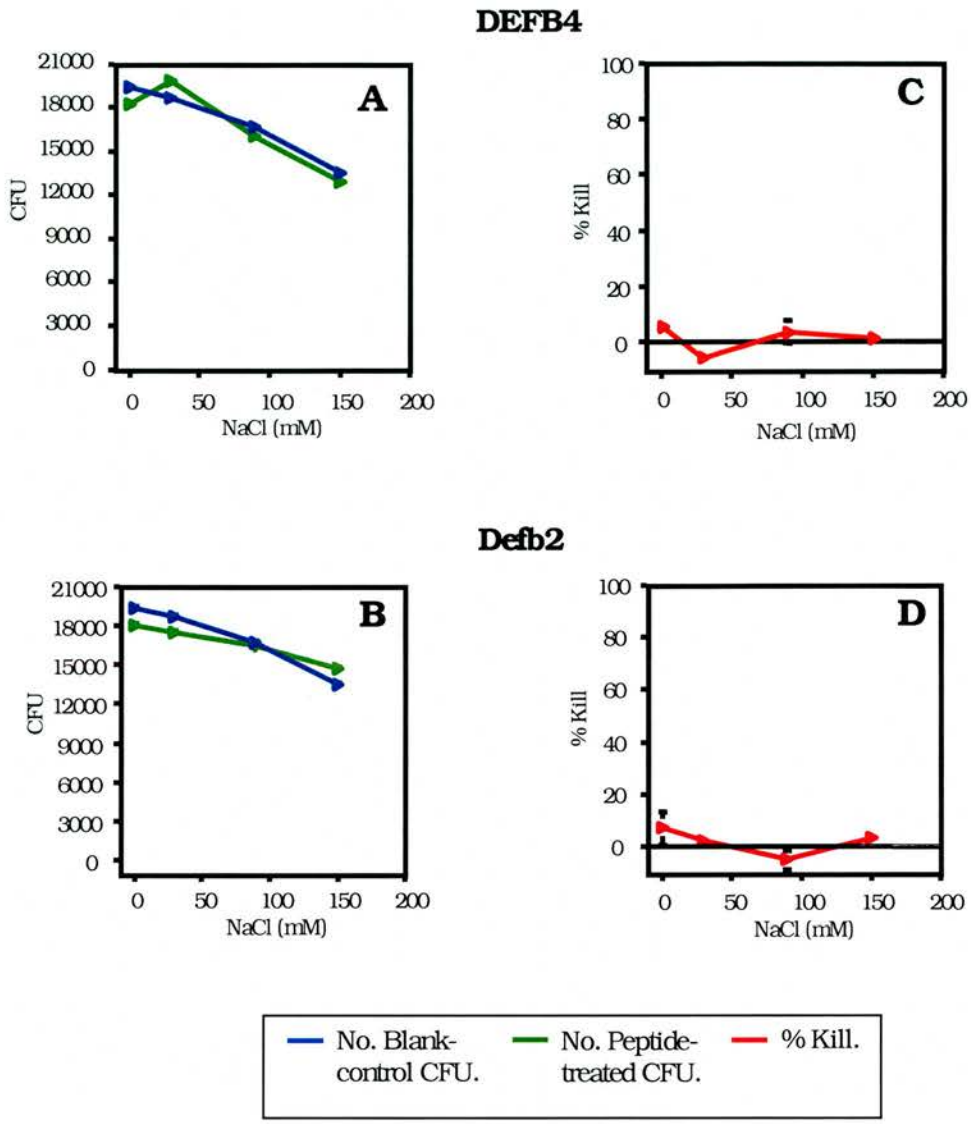

FIGURE 3.9: Antibacterial activity of DEFB4 and Defb2 against *Burkholderia cenocepacia* J2315.

Bacteria were incubated with the peptide at 50 μg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

3.3 Antibacterial Activity of Defr1

The identification of defensin-related 1 (Defr1), a β-defensin that lacks the first canonical cysteine raises interesting questions about the function of a peptide that diverts so dramatically from the typical β-defensin structure. Defr1 was synthesised and its antibacterial activity was assessed as for DEFB4 and Defb2. The activity of Defr1 was tested in comparison with a newly synthesised batch of Defb2 (and for the sake of comparison only termed Defb2^{2nd}) as a control this peptide was made using the same amino acid sequence that was used for the original batch Defb2. These peptides were tested against *Staphylococcus aureus* CF clinical isolate C1705, *Pseudomonas aeruginosa* CF clinical isolates J1385 and laboratory strain PAO1, *Burkholderia cenocepacia* CF clinical strain, epidemic strain J2315 and *Escherichia coli* clinical isolates J2408. Again, 50 μg/ml of peptide was used as standard. Studies were tested for reproducibility in a minimum of three experiment (*i.e.* n-3) and representative experiments are illustrated below in Figures 3.10–3.17.

3.3.1. Antibacterial activity of Defr1 against *S. aureus* C1705

Defr1 was observed to have statistically significant ($p < 0.01$) salt-sensitive activity against *S. aureus* C1705 at 0, 30 and 90 mM NaCl (Figure 3.10 B and D). The activity was maximal in the absence of NaCl, where 100% of bacteria were killed; this was reduced to 80% in the presence of 30 mM NaCl and further reduced by incubation with higher concentrations of NaCl. Thus, antibacterial activity was 50% in 90 mM NaCl and almost ablated at 150 mM. The activity of the second batch of Defb2 (Defb2^{2nd}), used as a control peptide, showed almost identical activity to the first batch of Defb2 (Figure

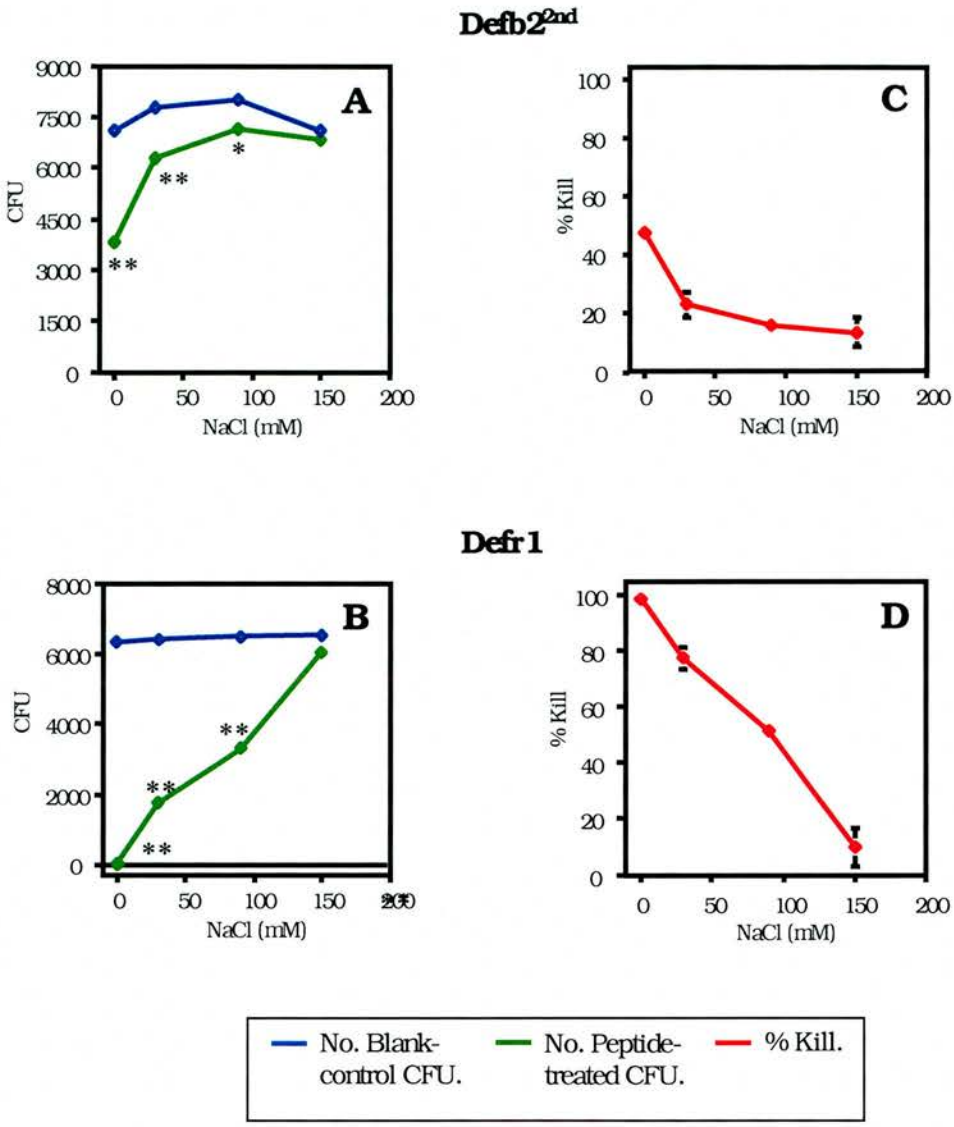

FIGURE 3.10: Antibacterial activity of Defb2^{2nd} and Defr1 against *Staphylococcus aureus* C1705.

Bacteria were incubated with the peptide at 50 µg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

3.10 A and C), with no statistically significant differences between the two sets of data. A comparison of the activities of Defr1 and Defb2^{2nd} shows that the antimicrobial activity of Defr1 against *S. aureus* C1705 was significantly greater than that shown by Defb2 at 0, 30 and 90 mM NaCl ($p < 0.01$). Indeed the activity of Defr1 against *S. aureus* was the highest level of activity of any of the peptides previously analysed (DEFB2, Defb2, DEFB1 and Defb1) using this method.

3.3.2 Antibacterial activity of Defr1 against *P. aeruginosa* J1385

Defr1 demonstrated highly potent and virtually salt-insensitive antibacterial activity against *P. aeruginosa* J1385 at 50 μg/ml over the full range of NaCl concentrations tested (Figure 3.11 B and D). Defr1 killed 100% at, 0, 30 and 90 mM the activity was slightly reduced only in the presence of 150mM with ≈95% of bacteria killed. This is the first peptide to show such high levels of activity. Defb2^{2nd} also showed statistically significant levels of salt-sensitive antibacterial activity ($p < 0.01$). This activity was maximal in 0 mM NaCl with 100% of bacteria being killed, this was reduced slightly to 90 % in the presence of 30 mM NaCl and further reduced to 75% and 20% in 90 and 150 mM NaCl respectively.

In comparison with the first-batch of Defb2, Defb2^{2nd} showed significantly ($p < 0.01$) greater levels of antibacterial activity at 0, 30 and 90 mM NaCl (Figure 3.10 A and C). The first batch of Defb2 demonstrated a maximum antibacterial activity of 60% in the absence of salt and this fell to 30% in 30 mM, 20 % in 90 mM and activity was almost ablated by the presence of 150 mM. These differences are very unusual as both peptides have the same

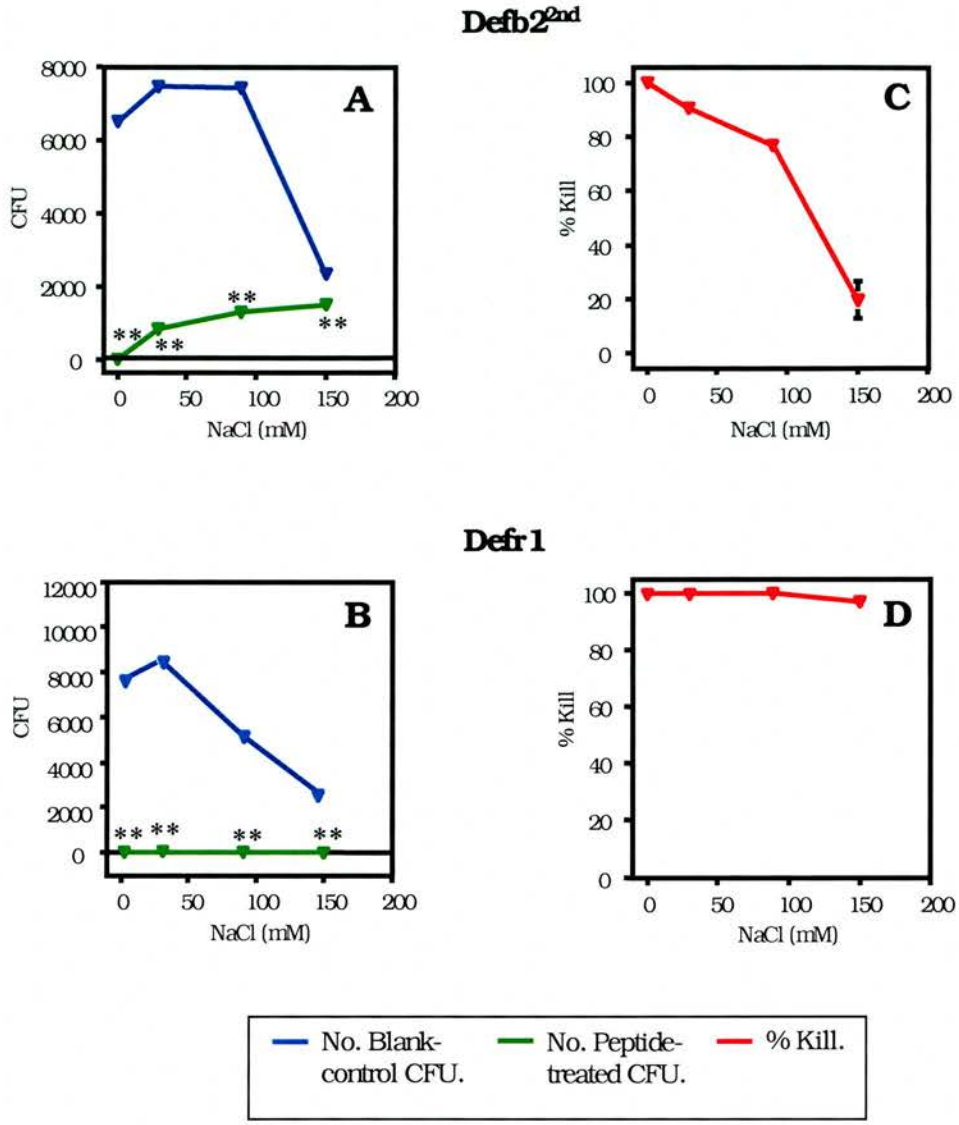

FIGURE 3.11: Antibacterial activity of Defb2^{2nd} and Defr1 against *Pseudomonas aeruginosa* J1385.

Bacteria were incubated with the peptide at 50 µg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

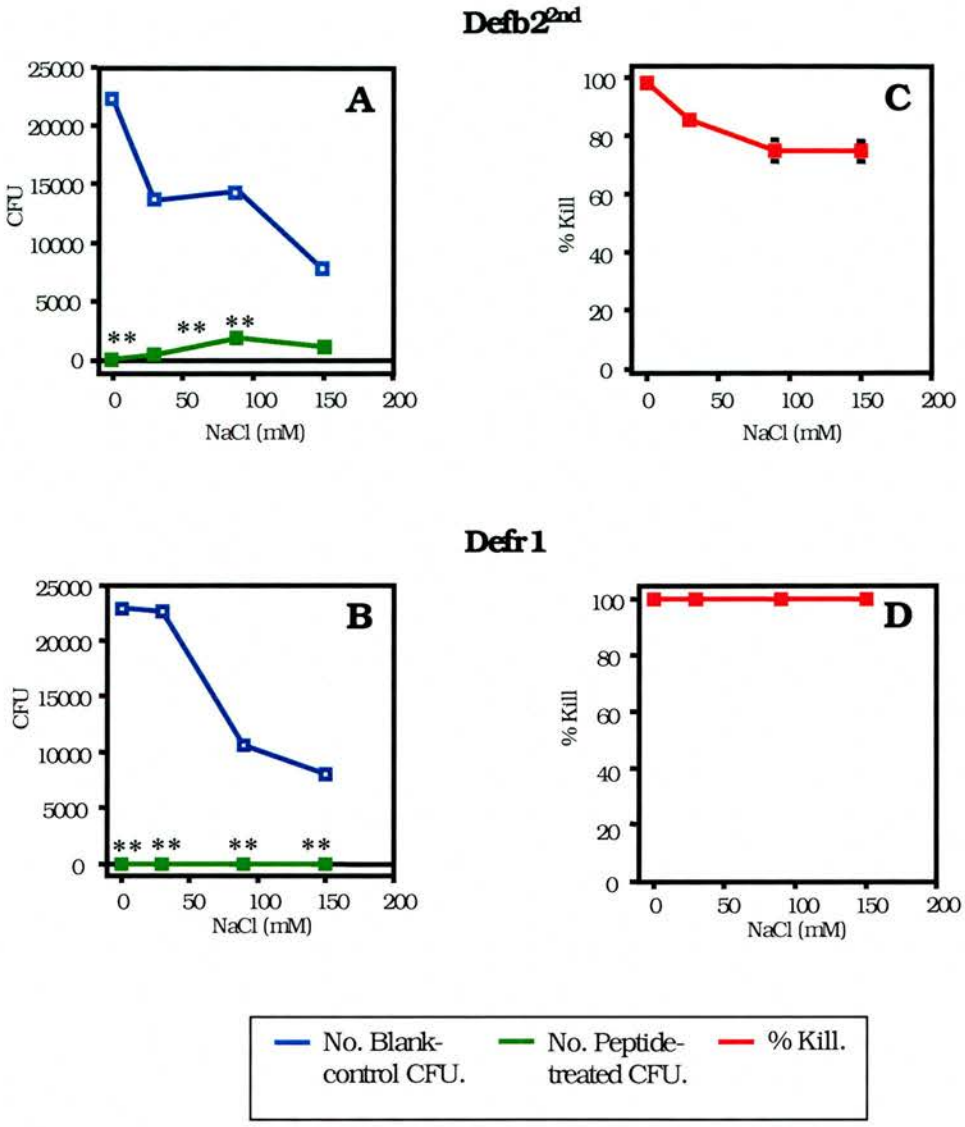

FIGURE 3.12: Antibacterial activity of Defb2^{2nd} and Defr1 against *Pseudomonas aeruginosa* PAO1.

Bacteria were incubated with the peptide at 50 µg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

amino acid composition, and therefore may represent differences in the folding of the two peptides.

3.3.3 Antibacterial activity of Defr1 against *P. aeruginosa* PAO1

Defr1 also showed very potent and statistically significant ($p < 0.01$) levels of activity against *P. aeruginosa* PAO1 (Figure 3.12 B and D). The levels of activity displayed were very similar to those against *P. aeruginosa* J1385 described above. Defr1 killed 100% of bacteria across the full NaCl concentration tested. Defb2^{2nd} also showed statistically significant levels of salt-sensitive antibacterial activity against *P. aeruginosa* PAO1 (Figure 3.12A and C). This activity of was greatest in the absence of NaCl and lowest at the 150 mM NaCl. Defb2^{2nd} killed 100% of bacteria at 0 mM and this was reduced to 85 % at 30 mM, the activity in 90 and 150 mM was fairly static with approximately 75% of bacteria killed. This activity was also greater than the activity shown against *P. aeruginosa* J1385. This is similar the relationship seen with the first-batch of Defb2 against *P. aeruginosa* PAO1 and J1385. However, the levels of activity of Defb2^{2nd} were again significantly higher than those observed with the first-batch of Defb2 at each NaCl concentration tested ($p < 0.01$).

3.3.4 Antibacterial Activity of Defr1 against *P. aeruginosa* PAO1: Effect of reduced peptide concentrations in high levels of salt.

At 50 μg/ml Defr1 demonstrates highly potent and salt-insensitive activity against *P. aeruginosa* PAO1 and J1385. The activity of Defr1 was assessed in 150 mM NaCl at peptide concentrations of 0, 5, 10, 25 and 50 μg/ml (Figure

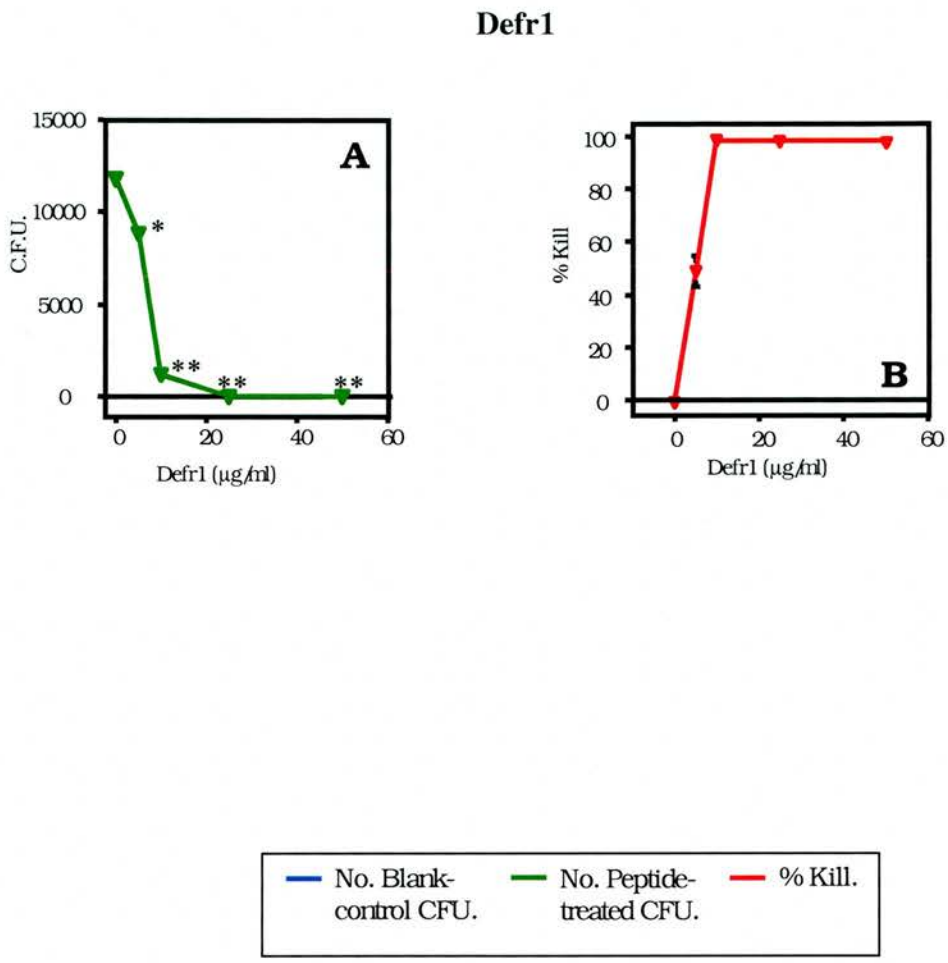

FIGURE 3.13: Antibacterial activity of Defr1 against *Pseudomonas aeruginosa* PAO1 at different peptide concentrations.

Bacteria were incubated with a range of peptide concentrations in 150 mM NaCl. Graph A shows the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graph B show the mean percentage kill ± standard error, this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

3.13A + B). Defr1 showed marginally greater activity against *P. aeruginosa* PAO1 compared to *P. aeruginosa* J1385 and consequently *P. aeruginosa* PAO1 was used in these experiments. Defr1 showed statistically significant activity at all peptide concentrations tested compared to the blank-control ($p < 0.01$). Maximal killing was observed at 50, and 25 μg/ml, where 100% of bacteria were killed, and at 10 μg/ml killing was reduced to ≈90%. When the Defr1 concentration was reduced to 5 μg/ml, killing was also reduced to approximately 50%. This result supports the previous data suggesting that Defr1 is a highly potent antimicrobial peptide against *P. aeruginosa* PAO1.

3.3.5 Antibacterial Activity of Defr1 against *P. aeruginosa* PAO1 at 5 μg/ml.

In 150 mM NaCl, at a concentration as low as 10 μg/ml Defr1 displays highly potent and apparently salt-insensitive activity, killing approximately 90% of bacteria. However, at a concentration of 5 μg/ml the activity is significantly reduced with only ≈50% of bacteria killed. The antimicrobial activity of 5 μg/ml of Defr1 was therefore examined over the normal range of salt concentrations to investigate the effect of the lower concentrations of NaCl on Defr1 activity (Figure 3.14 B and D). At NaCl concentrations of 0, 30 and 90 mM Defr1 demonstrated statistically significant ($p < 0.01$), highly potent and salt-insensitive antimicrobial activity, killing 100% of bacteria ($p < 0.01$). In 150 mM, killing was reduced to approximately 50% ($p < 0.05$) in agreement with the previous findings. The antimicrobial activity of Defb2^{2nd} was also assessed at 5 μg/ml. However, no significant antibacterial activity was observed at this concentration (Figure 3.14 A and C).

3.3.6 Antibacterial Activity of Defr1 against *E. coli* J2408

Defr1 demonstrated a statistically significant antibacterial effect against *E. coli* J2408 (Figure 3.15B and D). The activity of Defr1 was maximal in the absence of NaCl; killing 100% of bacteria and the activity was reduced by increasing the concentration of NaCl. In the presence of 30 mM NaCl, activity was reduced to ≈50%. A NaCl concentration of 90 mM reduced activity further still, killing approximately 30% of bacteria. The lowest level of activity was observed at 150 mM, at this concentration of NaCl activity was significantly reduced with ≈10% of a bacteria killed. The antimicrobial activity of Defr1 was statistically significant at 0 and 30 mM NaCl ($p < 0.01$) and 90 mM NaCl ($p < 0.05$). Defb2^{2nd} did not display antimicrobial activity against *E. coli* J2408. This supports the data obtained with the first-batch of Defb2 (Figure 3.15A + C).

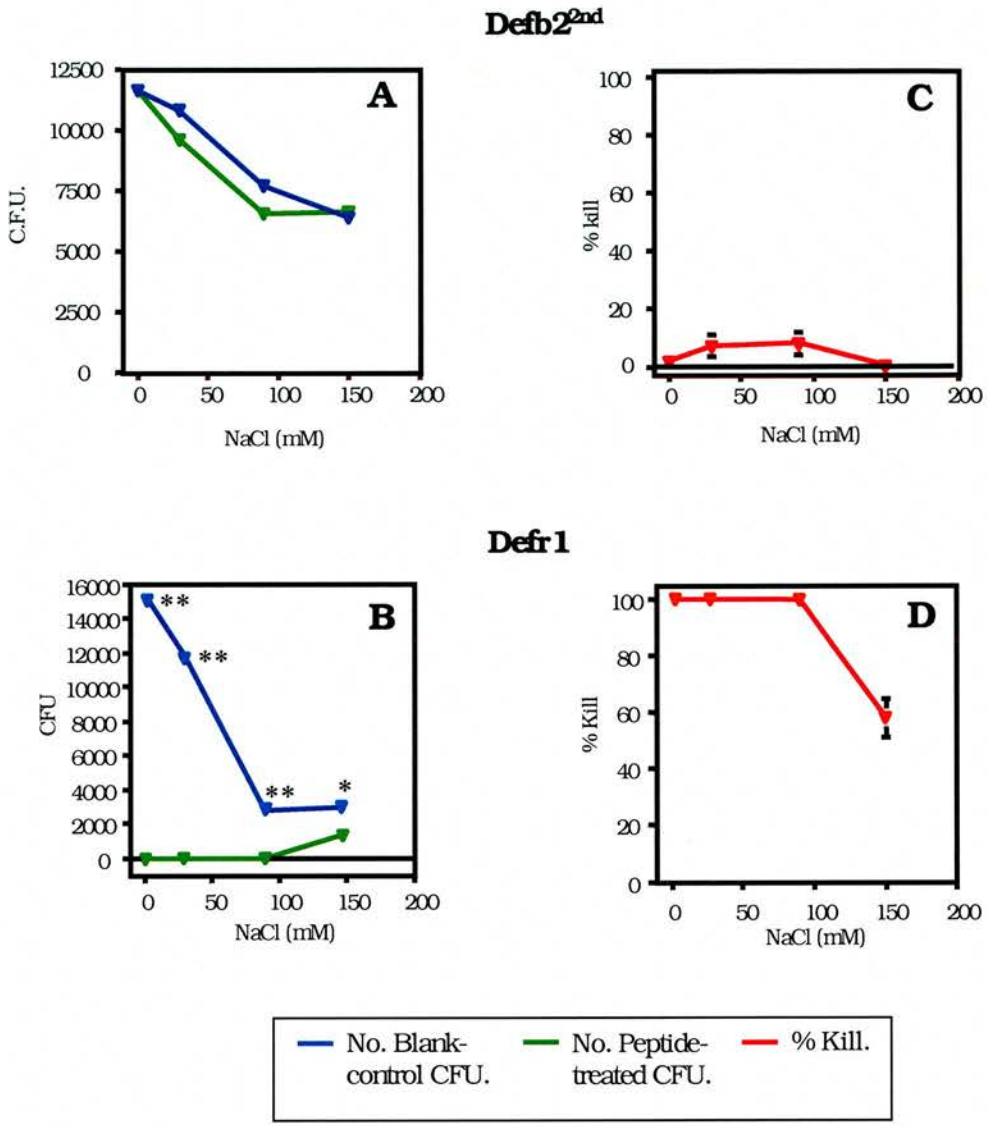

FIGURE 3.14: Antibacterial activity of Defb2^{2nd} and Defr1 against *Pseudomonas aeruginosa* PAO1 at 5 μg/ml.

Bacteria were incubated with the peptide at 50 μg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

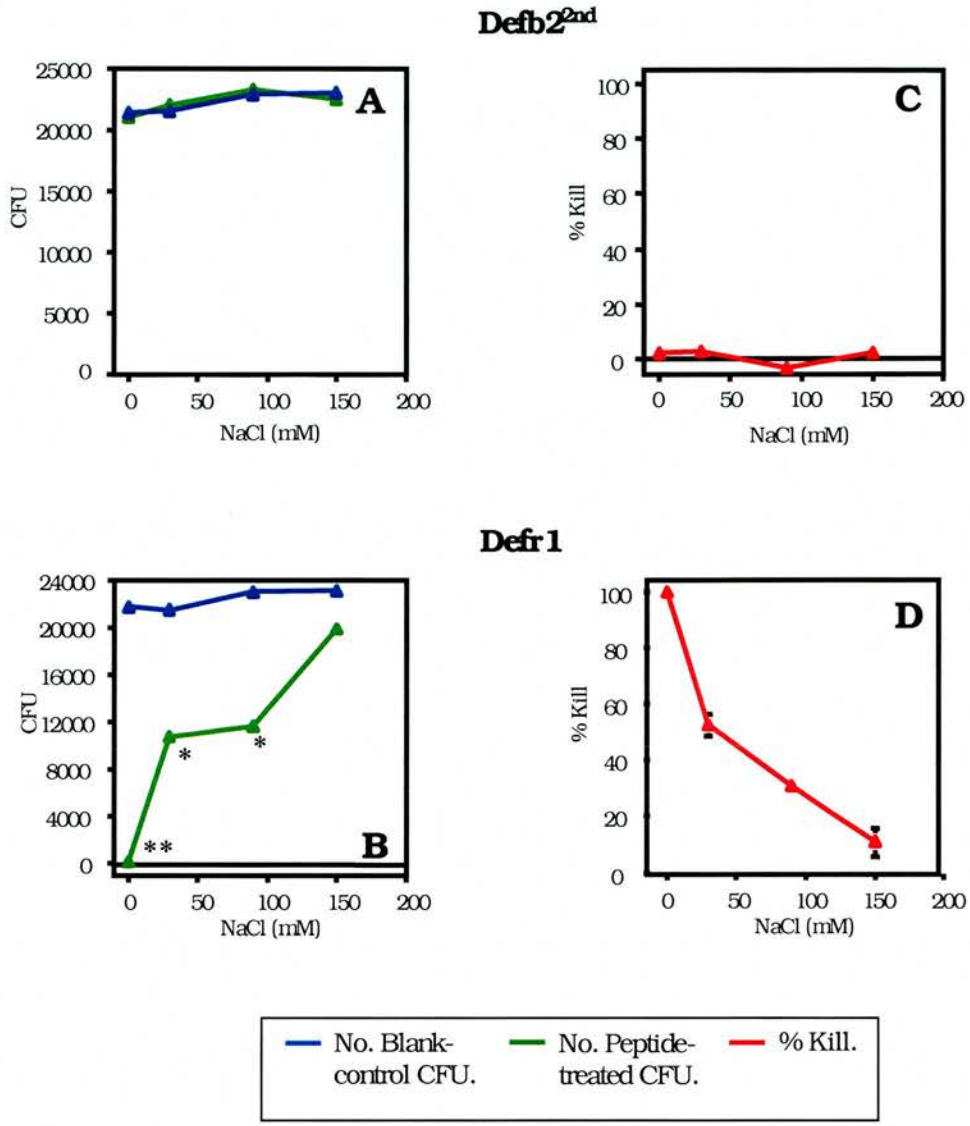

FIGURE 3.15: Antibacterial activity of Defb2^{2nd} and Defr1 against *Escherichia coli* J2408.

Bacteria were incubated with the peptide at 50 µg/ml over a range of salt concentrations. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. Experiments were repeated three times and the above is a representative experiment (n=3).

3.3.7 Antibacterial Activity of Defb2^{2nd} against *E. coli* J3201

The activity of Defb2^{2nd} against *P. aeruginosa* J1385 and PAO1 has varied in comparison to the response observed with the first-batch. To assess if the activity of the two Defb2 batches varied further, the activity of Defb2^{2nd} was also tested against *E. coli* J3201 (Figure 3.15 A and B). The first-batch of Defb2 demonstrated a low level of antibacterial activity against this strain, killing ≈25% of bacteria in the absence of NaCl. Defb2^{2nd} showed very similar levels of activity; in 0 and 30 mM NaCl Defb2^{2nd} demonstrated statistically significant levels of activity, killing approximately 25% and 15% of bacteria respectively and at 90 and 150 mM NaCl concentrations activity was ablated. This follows the same pattern as the activity displayed by the first-batch of Defb2.

3.3.8 Antimicrobial Activity of Defr1 against *B. cenocepacia* J2315 - Culture on *B. cepacia*-Selective Agar

Surprisingly, Defr1 also demonstrated statistically significant antibacterial activity against *B. cenocepacia* J2315, a bacterium previously found to be resistant to the activity of antimicrobial peptides (Morrison *et al.* 1998 and Hancock, 1997) (Figure 3.16 B and D). This is one of the first reports of a β-defensin active against *B. cenocepacia* J2315. Killing was maximal in the absence of NaCl, with ≈45% killing ($p < 0.01$), this was reduced to approximately 20% in 30 mM NaCl ($p < 0.05$), but killing was ablated by NaCl concentrations of 90 and 150 mM. No killing was observed with

Defb2^{2nd} against *B. cenocepacia* (Figure 3.16A and C), matching the response seen with the original batch of Defb2.

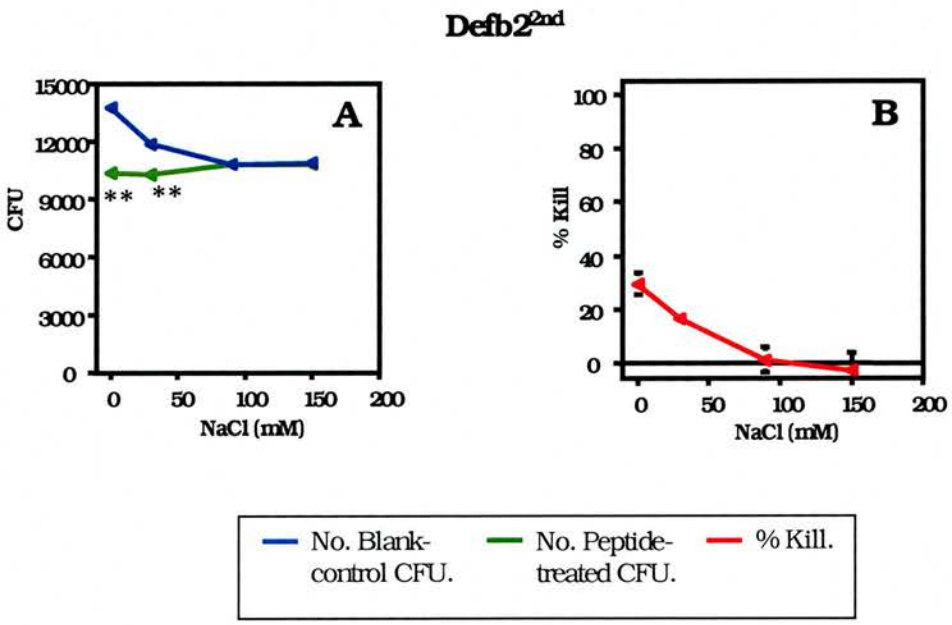

FIGURE 3.16: Antibacterial activity of Defb2^{2nd} against *Escherichia coli* J3201.

Bacteria were incubated with the peptide at 50 μg/ml over a range of salt concentrations. Graph A shows the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graph B shows the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. Experiments were repeated three times and the above is a representative experiment (n=3).

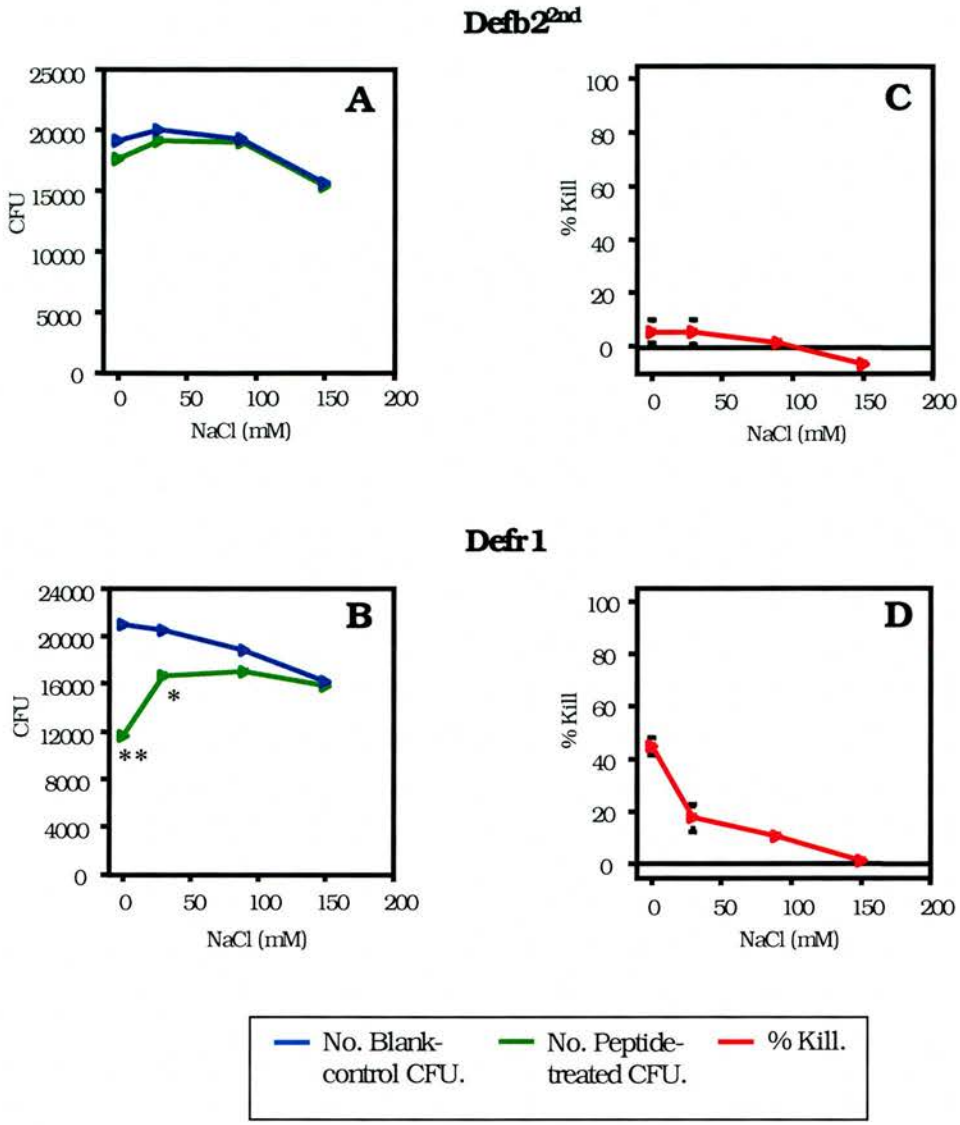

FIGURE 3.17: Antibacterial activity of Defb2^{2nd} and Defr1 against *Burkholderia cenocepacia* J2315. Culture on *B. cepacia*-selective agar.

Bacteria were incubated with the peptide at 50 μg/ml over a range of salt concentrations. Following incubation the bacteria were plated out on B. cepacia selective agar as detailed in the materials and methods. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

3.3.9 Antimicrobial Activity of Defr1 against *B. cenocepacia* J2315 - Culture on Nutrient Agar

The experiments described in section 3.3.8 were performed using selective agar to support the growth of *B. cenocepacia* J2315. This agar contains two antibiotics (polymyxin B and ticarcillin) to select for the growth of all bacterial strains of *B. cepacia* complex, which are resistant to these antibiotics. To confirm that the antimicrobial activity against *B. cenocepacia* J2315 was due to Defr1 alone and not due to an interaction of the antibiotics with Defr1, the experiments were repeated using nutrient agar with no selection. In the absence of the selective antibiotics, however, Defr1 killed in the same pattern as was previously seen with the selective agar (Figure 3.17 A and C). Moreover, 50 μg/ml (7790 Units/mg) of Polymyxin B, another antimicrobial peptide, failed to kill *B. cenocepacia,* this agrees with previous studies that have found *B. cenocepacia* to be resistant to this antibiotic (Figure 3.17 B and D). This result suggests that the experimental procedure does not render *B. cenocepacia* J2315 susceptible to antimicrobials to which it is normally resistant.

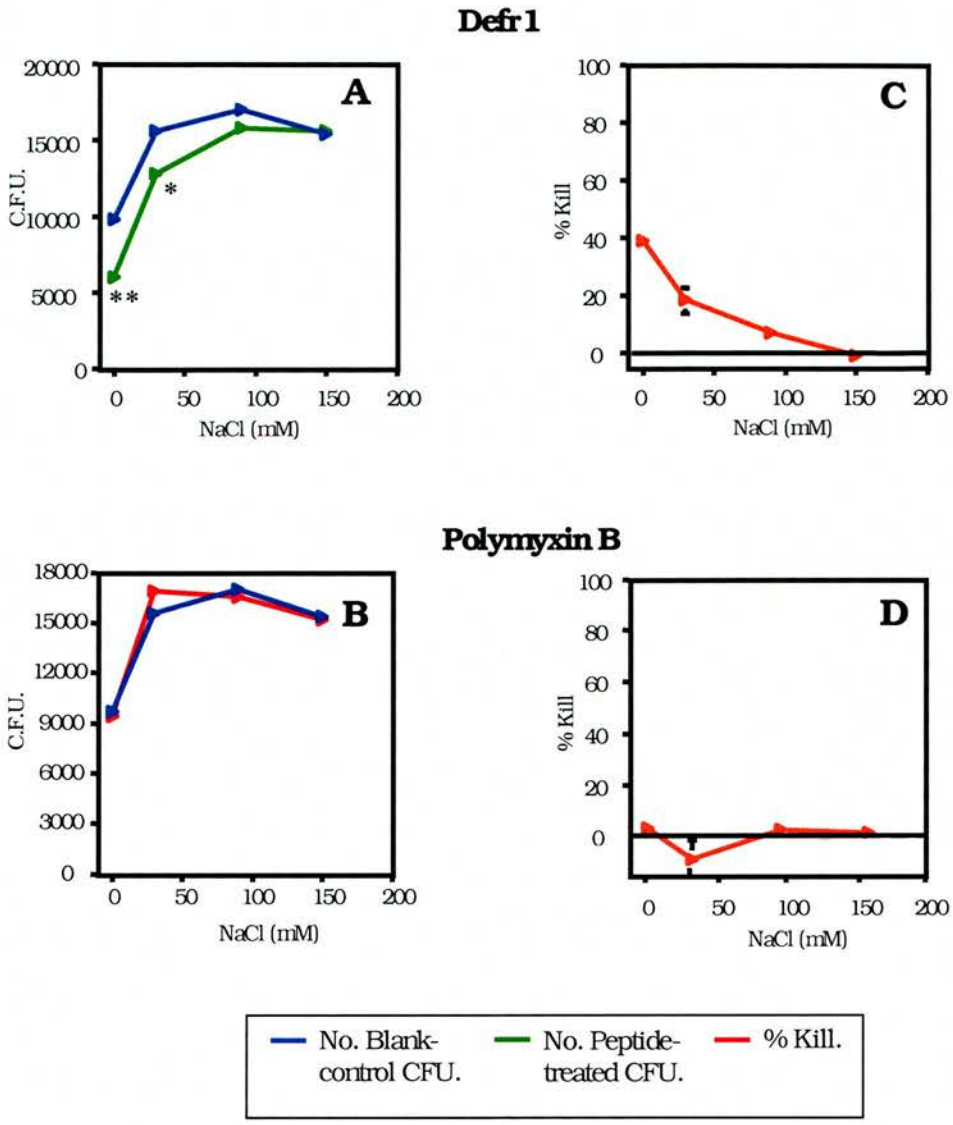

FIGURE 3.18: Antibacterial activity of Defr1 and polymyxin B against *Burkholderia cenocepacia* J2315. Culture of nutrient agar.

Bacteria were incubated with the peptide at 50 μg/ml over a range of salt concentrations. Following incubation the bacteria were plated out on *B. cepacia* selective agar as detailed in the materials and methods. Graphs A and B show the number of colony forming units (CFU) ± standard error from the blank-control and peptide-treated samples. Graphs C and D show the mean percentage kill ± standard error; this is assessed by calculating the number of CFU surviving in the peptide-treated sample as a percentage of the counts from the blank-control sample. ** = $p < 0.01$, * = $p < 0.05$. Experiments were repeated three times and the above is a representative experiment (n=3).

3.4 Discussion

3.4.1 Introduction

Many epithelial tissues such as the epidermis of the eye, the surfaces of the airways, and the digestive, reproductive and urinary tracts are exposed to the environmental microbes and are therefore potentially prone to colonisation. However, these epithelial surfaces are protected, in part, by the production of a variety of cationic antimicrobial peptides reviewed in (Zasloff, 2002). These peptides typically contain between 15 and 50 amino acids, are amphipathic in structure and have a net positive charge due to the presence of a high number of lysine and arginine residues. The α-defensins are expressed mainly in the granules of neutrophils but are also in the crypts of the small intestine. The β-defensins are produced by epithelial tissues such as the skin, salivary glands and urogenital and respiratory tracts (Bals, 2000). However, whilst the function and activity of α-defensins has been well studied, the activity of the β-defensins is less well characterised. They came to wider interest when the salt-sensitive nature of their activity was implicated in the pathogenesis of CF lung disease (Goldman *et al.*, 1997). It has been shown that primary cultures of CF airway epithelial have elevated NaCl concentrations in the airway surface liquid (ASL) and this results in a reduced ability to kill bacteria (Smith *et al.*, 1996; Zabner *et al.*, 1998). Moreover, it has also been demonstrated that synthetic human β-defensin 1 (DEFB1) has salt-sensitive antibacterial activity and is an important constituent of innate defence in the ASL (Goldman *et al.*, 1997). These results suggest that the loss of CFTR elevates NaCl concentrations and reduces the antibacterial activity and that this may leave the lungs of CF individuals prone to bacterial infection and colonisation, however evidence in support of

this theory remains largely absent. Furthermore, the β-defensins are viewed as 'natural antibiotics' and in the view of the increasing bacterial resistance to standard antibiotics, the study of antibacterial activity and role in the innate immune system of defensins is of great interest (Diamond, 2001).

	S. aureus C1705		*P. aeruginosa* J1385		*P. aeruginosa* PAO1	
Peptide	**%Kill^0**	**NaCl^{Max}**	**%Kill^0**	**NaCl^{Max}**	**%Kill^0**	**NaCl^{Max}**
DEFB4	80	90	100	90	100	150
Defb2	40	90	60	30	85	30
Defb2^{2nd}	45	90	100	150	100	150
Defr1	100	90	100	150	100	150

	E. coli J2408		*B. cenocepacia* J2315	
Peptide	**%Kill^0**	**NaCl^{Maa}**	**%Kill^0**	**NaCl^{Max}**
DEFB4	0	0	0	0
Defb2	0	0	0	0
Defb2^{2nd}	0	0	0	0
Defr1	100	150	45	30

Table 3.1: Table showing the spectrum of antimicrobial displayed by synthetic β-defensins at 50 μg/ml. The %Kill^0 represents the maximum percent-kill displayed by each peptide at 0 mM NaCl. NaCl^{Max} indicates the maximum NaCl concentration at which that peptide displayed statistically significant antimicrobial killing ($p < 0.05$ or 0.01).

3.4.2 Antibacterial Activity of Synthetic DEFB4

The data presented in this chapter (Summarised in Table 3.1) are consistent with previous observations that β-defensins possess salt-sensitive antimicrobial activity against Gram-negative and Gram-positive bacteria. DEFB4, Defb2 and Defr1 all showed – to varying degrees –reduced activity in the presence of NaCl (Table 3.1). These studies found the greatest activity for DEFB4 against *P. aeruginosa* compared to all other bacteria tested. DEFB4 killed 100% of *P. aeruginosa* J1385 and PAO1 in salt-free and 30 mM NaCl buffer, however activity was reduced by incubation with 90 mM and 150 mM NaCl. DEFB4-mediated killing of laboratory strain *P. aeruginosa* PAO1 was more resistant to the effects of elevated salt than killing of the CF isolate *P.*

aeruginosa J1385. For example, in the presence of 150 mM NaCl killing of *P. aeruginosa* J1385 was almost completely ablated, whereas significant activity was still observed against *P. aeruginosa* PAO1. DEFB4 also showed significantly elevated antibacterial activity against the mucoid-convertant *P. aeruginosa* J1532 compared to the non-mucoid *P. aeruginosa* J1385. These two bacteria are the same strain (confirmed by pulsed field gel electrophoresis; C. Doherty, personal communication). Therefore, this may suggest that the conversion to the mucoidy phenotype results in reduced resistance to DEFB4. This finding contrasts with a recent study that found that conversion to the mucoid form of growth in *P. aeruginosa* PAO1 is associated with activation of genes for resistance to antibiotics such as the cationic antibiotic tobramycin (Whiteley *et al.*, 2002). However, mucoidy *P. aeruginosa* are normally protected from the environment by production of a biofilm. However, in the experiments presented in this is thesis the bacteria were resuspended in a solution, which may have prevented production of an effective biofilm.

DEFB4 was also found to have significant antibacterial activity against *S. aureus* CF isolate C1705 in low salt, however, this activity was ablated by salt at a concentration of 150 mM, and in a NaCl concentration of 90 mM only ≈40% of bacteria were killed. No significant activity was observed for DEFB4 against *E. coli* clinical isolate J2408 and only a low level of activity was recorded against *E. coli* J3201, where 25% of bacteria were killed in the absence of salt. DEFB4 also failed to kill *B. cenocepacia* J2315, this result is consistent with previous findings that this bacteria, indeed the *B. cepacia* complex, is highly resistant to the activity of antimicrobial peptides (Hancock, 1997a).

These results generated using synthetic DEFB4 are in broad agreement with those from previously reported findings. The peptide used in one of these studies (Bals *et al.*, 1998b) was generated by a baculovirus system, which produced peptides of two different lengths due to variations in cellular processing. The most predominant form was a peptide of 41-amino acids, which corresponded to the sequence of the mature peptide initially isolated from skin (Harder *et al.*, 1997), and this also appears to be the predominant form in the ASL. A 38-amino acid peptide, missing 3 amino acids at the NH_2-terminal, was also isolated; however, no significant difference was observed between the activity of the two peptides (Bals *et al.*, 1998b), and the 38 amino acid peptide is used in the studies described in this chapter. The Bals *et al.* study found activity against *P. aeruginosa* and *S. aureus* using recombinant-derived DEFB4 with a minimum inhibitory concentration (MIC) of 62 μg/ml, however their study also reporter an inhibitory effect of DEFB4 on *E. coli* D31, whereas the current work found no activity against *E. coli* J2408. Another study observed much greater levels of activity of DEFB4 against *P. aeruginosa* with a LD_{90} (concentration required to reduce bacterial survival by 90%) of near 10μg/ml and it is interesting to note that this study used the 41-amino acid peptide (Harder *et al.*, 1997). Against the Gram-positive *S. aureus,* however, DEFB4 showed only bacteriostatic activity at relatively high concentrations (100 μg/ml). It is not stated how the peptide used in the Harder *et al.* (1997) study was generated, nor how the antibacterial activity was assessed, so it is possible that the recorded differences are due to experimental-design differences or variations in the production and folding of the peptide. Moreover, it is difficult to compare the results between these different studies as the methods of data presentation (% kill, MIC and LD_{90}) differ markedly.

A further study also assessed the antibacterial activity of DEFB4 and reported salt-sensitive antibacterial activity (Singh *et al.*, 1998). This work assessed bacterial viability using an *E. coli* expressing a luminescence protein. Interestingly, they found the recombinant DEFB4 used in their study to be far more active than in the studies reported here, although it is possible that the strains of bacteria used are more sensitive. In their assay, recombinant DEFB4 was able to kill approximately 100% of bacteria in the presence of 75 mM NaCl at a concentration of only 0.5 μg/ml. In the studies described in this chapter, synthetic DEFB4 showed no activity against *E. coli* J2408 and only weak activity against *E. coli* J3201 in low salt and at a concentration of 50 μg/ml. A similar trend was also found with recombinant DEFB1 compared to the results obtained by Dr Donald Davidson from this laboratory using synthetic DEFB1 (Morrison *et al.*, 1998; Morrison *et al.*, 2002b). Moreover, Singh *et al* also reported that DEFB4 was more active than DEFB1 against *E. coli* and *P. aeruginosa* PAO1 in 10 mM NaCl; these findings were not repeated with synthetic DEFB4 or DEFB1 in this study. Furthermore, DEFB4 showed no activity against *E. coli* J2408, whereas DEFB1 was found to kill ≈50% of *E. coli* J2408 in the absence of salt. However, whilst both DEFB4 and DEFB1 showed potent activity against *P. aeruginosa* PAO1 in low levels of NaCl (both killed 100% of *P. aeruginosa* PAO1 in the absence of salt); DEFB4 maintained highly potent killing activity in 30 mM, whereas DEFB1 activity was greatly reduced by high levels of salt.

The failure of the DEFB4 used in these studies to show activity against *E. coli* J2408 and only weak activity (25% killing in salt-free phosphate buffer) against *E. coli* J3201 may be due to strain differences such as their origins, or experimental differences. However, it is unusual as antibacterial activity was

reported for DEFB4 by two earlier studies (Bals *et al.*, 1998b; Singh *et al.*, 1998). As mentioned earlier, the MIC of recombinant DEFB4 against *E. coli* D31 was reported to be 62 μg/ml (Bals *et al.*, 1998b). Singh and colleagues, however, found strong activity at 0.1 μg/ml DEFB4 in low concentrations of NaCl against *E. coli* DH5α. Another study by Tomita *et al.* (2000) also using synthetic DEFB4 found activity against five different *E. coli* strains at 20 μg/ml or less (Lehrer and Ganz, 1996). The high levels of activity found in the Singh *et al.* system and the activity reported by Bals *et al.* and Tomita *et al.* against *E. coli* suggests that DEFB4 does indeed possess antimicrobial activity against *E. coli*. The discrepancy between these findings and the current study may be due to the different bacterial strains, peptide production methods or the assays used. For example, the incubation time used in the other studies discussed was considerably longer than the 30 min used in the studies presented in this chapter. Moreover, the other studies on DEFB4 used a 41-amino acid peptide that was three residues longer at the NH_2-terminus than the synthetic peptide used in this study, and this may have affect its activity. Although, Singh *et al.* report no difference in the activities of the peptide variants, another study on natural variants of DEFB1 found in urine did find significant differences in the activity of peptides of different lengths. The 36 amino acid variant of DEFB1 was shown to have greater antimicrobial activity against *E. coli* compared to the longer peptide varieties (37 and 42 residues) (Valore *et al.*, 1998). The murine orthologue of DEFB4 is Defb4, it would be predicted that these two peptides would show similar levels and spectra of activity, however, no functional data has yet been published for Defb4 and therefore comparison is impossible.

3.4.3 Antibacterial Activity of Defb2

This chapter includes novel data on the antimicrobial activity of synthetic murine β-defensin 2, Defb2 (Table 3.1). Defb2 demonstrated lower levels of activity compared to DEFB4 against *S. aureus* CF isolate C1705, and *P. aeruginosa* CF isolate J1385 and laboratory strain PAO1. The finding that DEFB4 and Defb2 show greater activity against *P. aeruginosa* PAO1 than *P. aeruginosa* J1385 is in agreement with previous findings with both DEFB1 and Defb1, which also found *P. aeruginosa* J1385 to be more resistant to β-defensins compared to *P. aeruginosa* PAO1 (Morrison *et al.*, 1998). *P. aeruginosa* J1385 is a virulent CF pathogen, whereas the PAO1 strain is a highly passaged laboratory strain. This may explain, at least in part, the different susceptibilities to defensins. As with DEFB4, Defb2 showed no activity against *B. cenocepacia* J2315, nor *E. coli* J2408, and only a low level of antimicrobial activity against *E. coli* J3201. Defb2 only showed significant antibacterial activity against the bacteria tested in the presence of NaCl concentrations of 30mM or less, this contrasts with DEFB4, which was observed to maintain statistically significant antibacterial activity ($P < 0.05$) in up to 90 mM NaCl against *P. aeruginosa* J1385 and PAO1 and *S. aureus* C1705. A similar pattern was also observed in the work using DEFB1 and Defb1 at 50 μg/ml against *P. aeruginosa* J1385 (Morrison *et al.*, 1998; Morrison *et al.*, 2002b). The findings in two different studies that mouse β-defensins 1 and 2 are less active than human β-defensins 1 and 2 against *P. aeruginosa* J1385, suggests that differences in the success of peptide folding are unlikely to be a factor, and may represent the existence of species-specific differences in the activities of β-defensins. It is possible that Defb1 and Defb2 have greater activity against a different range of bacteria than DEFB1 and DEFB4. The

pathogens used in this study, such as *P. aeruginosa* J1385, are human pathogens and rarely colonise mice; indeed established *P. aeruginosa* infections in CF mouse models suggest that mice are not particularly susceptible to colonisation with *P. aeruginosa* (McCray *et al.*, 1999; Larbig *et al.*, 2002). Therefore, murine antimicrobial peptides, such as the β-defensins, may not have evolved activity against these bacteria, but rather against bacteria that are pathogenic in mice and relevant to their ecological niche. It would be of great interest to investigate the relative activity of human and murine β-defensins against pathogens known to cause infections in mice.

3.4.4 Antibacterial Activity of Defb2^{2nd}

A second batch of Defb2 (solely for comparison purposes referred to as Defb2^{2nd}) was also synthesised to use as a control in the analysis of Defr1 function (see later). This peptide gave the same spectrum of activity as the first batch of Defb2 against *S. aureus* C1705 and *E. coli* J3201, and like the first batch of Defb2 also showed no activity against *B. cenocepacia* J2315 and *E. coli* J2408 (Table 3.1). Surprisingly, however, Defb2^{2nd} demonstrated significantly different spectra of activity against the two previously tested strains of *P. aeruginosa* (Table 3.1). Defb2^{2nd} had much higher antimicrobial activity against *P. aeruginosa* PAO1 and J1385 than was originally observed for Defb2; in both cases the activity was more similar to the spectrum of activity seen for DEFB4 against the same bacteria. Against *P. aeruginosa* J1385 in the absence of NaCl, Defb2^{2nd} killed 100% of the bacteria, this contrasts with 40% of bacteria killed in experiments using the first batch of Defb2. Furthermore, whereas the original Defb2 peptide showed no significant killing in the presence of 90 or 150 mM NaCl, Defb2^{2nd} killed approximately 75% of bacteria in 90 mM NaCl and ≈25% of bacteria in 150 mM NaCl. A different

set of results was also obtained for Defb2^{2nd} against *P. aeruginosa* PAO1 compared those originally recorded with Defb2. The first batch of Defb2 showed the greatest activity in the absence of NaCl, killing ≈80% of bacteria, whereas Defb2^{2nd} killed 100% of bacteria at the same NaCl concentration. In the presence of 90 or 150 mM NaCl, Defb2^{2nd} maintained significant activity, killing approximately 75% of bacteria, whereas the original Defb2 show no reproducible significant activity at 90 or 150 mM NaCl. The bacteria used in the two studies were cultured and handled in an identical fashion and were certified as the appropriate strain and were confirmed to be identical to the isolate used in the initial experiments by restriction endonuclease digestion (personal communication, C. Doherty, University of Edinburgh). Therefore, it is unlikely that the different activities were due to differences in the bacterial strains used. The differences may however be due to differences in the production of the two peptides. Albachem Ltd. (Edinburgh, UK) analysed the structure of the two peptides by MALDI TOF mass spectrometry. Analysis of the HPLC spectra produced for the two peptides reveals that whereas Defb2^{2nd} produced a single main peak suggesting the existence of only one form of the peptide, the analysis for the original batch of Defb2 shows the presence of two large peaks (See Appendix). Whilst one peak corresponds to the peptide also seen in Defb2^{2nd} analysis. A second peak indicates the presence of a substantial quantity of Defb2 in which the methionine at position 38 has become oxidised (Figure 3.1). The effects of this on the structure of Defb2 are unknown. However, the reduced antimicrobial activity of this batch of Defb2 compared to Defb2^{2nd} may suggest that the result is a less active form of the peptide against *P. aeruginosa,* although the activity of Defb2 against the other strains of bacteria tested was not affected by this change. However, with the exception of *P. aeruginosa,* the results obtained with Defb2^{2nd} are in agreement with the

activities observed with the original batch of Defb2, suggesting that these results are reproducible. Nevertheless, the differences observed against *P. aeruginosa* may suggest that alterations in the peptide folding can lead to large changes in the activity of the peptide and thus stresses the importance of peptide folding and composition. It is noteworthy that even after taking into account the increased anti-pseudomonal activity of Defb2^{2nd}, the results still indicate that murine Defb2 is less effective against the tested pathogens than human DEFB4. Although it is important to note when comparing the results of these two peptides that DEFB4 and Defb2 are not functional orthologues, and may therefore have evolved quite separately with specificity against different pathogens.

The results presented here are in broad agreement with previous studies on the activity of murine β-defensins. In comparison with synthetic Defb1, Defb2 shows similar levels of activity against *S. aureus* C1705, both peptides killed 75-80% of bacteria in the absence of salt and the activity of both β-defensins was ablated by high levels of salt. However, Defb1 showed weak antimicrobial activity against *E. coli* J2408, whereas Defb2 failed to kill this organism and showed only weak activity against a second *E. coli* strain, J3201. Moreover, both batches of Defb2 showed greater activity against *P. aeruginosa* J1385 than did Defb1, which killed approximately only 40% of bacteria in the absence of salt (Morrison *et al.*, 1998), suggesting that Defb2 has antimicrobial activity against a different range of bacteria than Defb1. A separate study on the antimicrobial activity of cell lysates from cell lines transfected with Defb1 cDNA showed that Defb1 possessed potent and salt-sensitive antimicrobial activity against *E. coli, S. aureus* and *P. aeruginosa* compared to untransfected cell lines (Bals *et al.*, 1998a). However, this study did not quantify or purify Defb1, so the level of Defb1 used in this study is

not known, and furthermore, the possibility that Defb1 interacted with other factors present in the cell lysates to increase its activity can not be ruled out. Mouse β-defensin 3 (Defb3) has been shown to possess highly potent antibacterial activity against P. aeruginosa PAO1 with an MIC of 8 μg/ml and also displays activity against *E. coli* D31 with an MIC of 16 μg/ml (Bals *et al.*, 1999). This suggests that Defb3 has potent activity against Gram-negative bacteria, whereas Defb2 activity does not appear to be potent against Gram-negative bacteria as it failed to kill *E. coli* and showed variable activity against *P. aeruginosa*.

3.4.5 Antibacterial Activity of Defr1

Defensin-related 1 (*Defr1*) is a novel β-defensin gene in which the first of the canonical cysteines that are present in all β-defensins previously isolated has been substituted for a tyrosine. As the loss of this residue would prevent the formation of a disulfide bond and therefore likely lead to large structural changes the antimicrobial activity of Defr1 was also assessed using a synthetic peptide (Morrison *et al.*, 2002a). Defr1 demonstrated salt-sensitive antimicrobial activity against *S. aureus* C1705 and *E. coli* J2408 (Table 3.1). In the absence of salt 100% of bacteria were killed, this is greater activity than was seen against either *S. aureus* C1705 or *E. coli* J2408 with any of the other peptides analysed in this chapter, however antimicrobial activity was reduced in 30 and 90 mM NaCl and ablated by 150 mM salt. Defr1 also demonstrated highly potent activity against *P. aeruginosa* J1385 and PAO1; these results suggest that the loss of the first canonical cysteine does not result in loss of antimicrobial activity (Table 3.1). This is also demonstrated in a previous study in which analogues of the α-defensin, HNP-1 engineered

to have one or two disulfide bridges maintain activity seen with the native peptide (Mandal and Nagaraj, 2002).

Defr1 exhibited its greatest activity against both *P. aeruginosa* strains compared to all other bacteria tested. The antimicrobial effect of Defr1 against *P. aeruginosa* J1385 was virtually salt-insensitive at 50 μg/ml and the activity was completely resistant to the effects of salt against *P. aeruginosa* PAO1 at the same concentration. In the presence of 150 mM NaCl the antimicrobial activity of Defr1 against *P. aeruginosa* J1385 was only marginally reduced to ≈95%, at all other NaCl concentrations, and in the case of *P. aeruginosa* J1385 across the full salt concentration range, killing was 100%. Previous studies have indicated that salt-sensitivity can be overcome by increasing the peptide concentration (Singh *et al.*, 1998). In this study, the concentration of DEFB1 and DEFB4 was increased to abrogate the effect of elevated NaCl. To further investigate the potency of Defr1 against *P. aeruginosa* the effect of lower concentrations of Defr1 on the survival of *P. aeruginosa* PAO1 was assessed at 150 mM NaCl. Defr1 maintained a high level of potency in concentrations as low as 25 μg/ml, with 100% of bacteria killed and even at a concentration of 10 μg/ml ≈90% of bacteria were killed. However, at a concentration of 5 μg/ml antibacterial activity was reduced to 50% killing. This suggests that Defr1 possesses significant antimicrobial killing at comparatively low concentrations, even in the presence of 150 mM NaCl. The full salt-sensitivity of Defr1 at 5 μg/ml was also assessed to investigate whether Defr1 possessed salt-insensitive activity at this concentration. At this concentration however, Defr1 still possessed highly potent antimicrobial activity in salt concentrations as high as 90 mM, killing 100% of bacteria in 0, 30 and 90 mM NaCl, but in 150 mM NaCl antibacterial activity was reduced to 50% in agreement with the previous study. By

comparison, Defb2^{2nd} showed no significant activity against *P. aeruginosa* at 5 μg/ml. These results suggest that Defr1 possesses highly potent anti-pseudomonal activity; the LD_{90} of Defr1 against *P. aeruginosa* PAO1 of 10 μg/ml and LD_{50} of 5 μg/ml compares highly favourably with the LD_{90} of 'near' 10 μg/ml for DEFB4 against *P. aeruginosa* (Harder *et al.*, 1997). Moreover, the LD_{90} of Defr1 is only slightly higher than the MIC of 4.1 μg/ml for DEFB104 also against *P. aeruginosa* PAO1, which is the lowest MIC observed to date for a β-defensin (Garcia *et al.*, 2001b) and is lower than the MIC (8 μg/ml) of Defb3 against *P. aeruginosa* PAO1 (Bals *et al.*, 1999). Interestingly, however, while the activity of Defr1 against *P. aeruginosa* is significantly lower than the MIC recorded for DEFB103 of 26.5 μg/ml, this peptide does demonstrate salt-insensitive killing of the Gram-positive *S. aureus* (Garcia *et al.*, 2001a). Greater analysis of the structure of these two peptides may help to elucidate the structural differences responsible for their *P. aeruginosa* (Defr1) and *S. aureus* (DEFB103) specificities. Furthermore, these results may further suggest the evolution of some β-defensins with specific, rather than broad antimicrobial activities (Schroder, 1999). It must be noted, however, many of these antimicrobial studies were performed under different experimental conditions, which make comparison difficult. Consequently, the MIC of Defr1 should be calculated using a standard Microbroth dilution method to make a more accurate comparison.

Defr1 expression has been detected in the heart, uterus and testis. This pattern of expression is quite limited and unique compared to that of the other β-defensins. However, a recently identified murine β-defensin, Defb6, was found to be expressed in skeletal muscle (Yamaguchi *et al.*, 2001) and a defensin-like molecule, Binb1, which possesses antimicrobial activity has been identified in rats and its expression appears to be confined to the caput

region of the epididymis (Li *et al.*, 2001). These findings demonstrate widespread distribution of the β-defensin (and β-defensin-like) gene expression. Many tissues express a multiplicity of β-defensins and their functions in these tissues may be, to some extent, overlapping. However, in other tissues specific β-defensins may form an important aspect of the immune system.

Perhaps the most startling result obtained with the Defr1 peptide was the finding that it has antimicrobial activity against *B. cenocepacia* J2315. This bacterium was previously thought to be resistant to the action of cationic peptides due to a nonreactive outer surface (Hancock, 1997b). However, synthetic DEFB103 was shown to have antimicrobial activity against *B. cenocepacia* ATCC17770 and also against an unspecified clinical isolate at a low concentration of the peptide (6.6 μg/ml) (Garcia *et al.*, 2001a). Synthetic Defr1 killed ≈45% of bacteria in the absence of salt, this was reduced by incubation in 30 mM, but 90 or 150 mM salt ablated antimicrobial activity. It is interesting to note that the *B. cenocepacia* CF isolate J2315 is the Edinburgh-Toronto epidemic strain, and is one of the most resistant to the action of conventional antibiotics (Nzula *et al.*, 2002). In comparison with Defr1, neither DEFB4 nor either batch of Defb2 demonstrated the ability to kill *B. cenocepacia.* In the above experiments, following incubation with Defr1 the surviving bacteria were plated out on *B. cepacia*-selective agar, which contains high levels of antibiotics (32.5 mg/ml polymyxin B and 100 μg/ml tircarcillin). Therefore, the experiments were repeated using nutrient agar without antibiotic-selection to investigate the possibility that the observed antibacterial activity was due the interaction of Defr1 and the antibiotics. These studies gave the same spectrum of results as was seen previously,

suggesting that the antimicrobial activity was due to the action of Defr1 alone; moreover in this set of experiments 500 μg/ml Polymyxin B, to which *B. cenocepacia* is resistant, was also included as a control and this failed to kill *B. cenocepacia*. This suggests that the observed activity is not an artefact of the experimental procedure.

It is unclear why Defr1 possesses activity against *B. cenocepacia* when other cationic peptides do not display such potent anti-pseudomonal activity. One possible explanation is that while many β-defensins show broad-range antibacterial activity, others have evolved more specific and defined activity against a narrow spectrum of bacteria (Schibli *et al.*, 2002; Schroder, 1999). Defr1 may have evolved highly potent anti-pseudomonal activity, as it has been argued that DEFB103 evolved highly specific activity against *S. aureus* (Garcia *et al.*, 2001a). The manner in which Defr1 is folded may also have lead to greater clustering of its positive residues compared to other β-defensins, and this may result in its increased potency as this clustering may increase the electrostatic interaction between peptide and target membrane (Yu *et al.*, 2000). Yu *et al.* (2000) have shown that α-defensins engineered to have a head-to-tail cyclic structure are more potent and less salt-sensitive than the native compound and proposed that this was due to clustering of positively charged residues. However, the activity seen by Defr1 may also be due to the loss of the cysteine and subsequent alterations in the β-defensin structure. However, the precise effect of the amino acid substitutions on the structure of the peptide remains unknown. The structure of β-defensins and its effect on antibacterial activity is discussed in detail below.

3.4.6 β-Defensin Structure

To date the structures of several different murine and human β-defensins have been analysed. A crystal structure of DEFB4 has been determined for two different forms that differed in the quaternary arrangement of the DEFB4 monomer (Hoover *et al.*, 2000). In both forms, however, the three-dimensional structure of the monomer was highly similar and were shown to include an N-terminus α-helix and three antiparallel β-sheets. In one form, a dimer was observed to be formed by interaction between the first β-sheets of each monomer. The second crystal structure was an octomer formed from four dimers. The authors proposed that it was likely that the dimer was the native form and the octomeric form of DEFB4 represents the form bound to bacterial membranes (Hoover *et al.*, 2000). This oligomerisation of DEFB4 contrasts with the structure of DEFB1, which showed no evidence of higher-order structures similar to the octomeric DEFB4 although dimers were identified (Hoover *et al.*, 2001). Despite this difference in the quaternary arrangements, the tertiary structure of DEFB1 was very similar to that for the DEFB4 monomer. Another study has determined the solution structure of DEFB1 and DEB2 using nuclear magnetic resonance (NMR) analysis and observed a highly similar secondary structure as compared to the crystal structure of DEFB1 and 2 but found only monomeric forms(Bauer *et al.*, 2001). Furthermore, the authors argue that the oligomerisation of DEFB4 observed in the crystal structure was an effect of the conditions used for crystallisation such as high local concentration. It is possible, therefore, that β-defensins exert their antimicrobial effects as oligomers. Interestingly, however, the recently determined solution structure of DEFB103 suggests

that it does exist as a dimer in solution, moreover this study saw DEFB1 and DEFB4 to exist in only monomeric form (Schibli *et al.*, 2002).

In order to elucidate the structure of Defb2^{2nd} and Defr1 Studies of the synthetic peptides have been conducted by Dr Perditta Barran at the Chemistry Department, University of Edinburgh. Analyses of Defb2^{2nd} reveal the presence of one monomeric form, which possesses three disulfide bonds. The analyses the Defr1 structure, however, reveal that it exists both in monomeric and dimeric forms. Further study confirmed that the monomer contained two disulfide bonds as would be expected for a molecule with five cysteines. However, the dimer was composed of two forms. One form possessed three disulfide bonds, whereas the alternate form had four, although the arrangements of the disulfide bonds are not known. Interestingly, a very recent study has identified a polymorphism in the DEFB1 gene that substitutes a serine for one of the conserved cysteines. The peptide, however, retained levels of antibacterial activity similar to the originally identified DEFB1 even though the cysteine-pairing is altered (Circo *et al.*, 2002).

Bauer *et al.*, (2001) have recently reported the structure for Defb8, a β-defensin that differs form Defr1 by only three amino acids, one of which gives the Defb8 peptide the six cysteine pattern conserved in other β-defensins. Moreover, Defb8 is 98% identical to Defr1 across the entire length of the gene, which suggest that these are different forms of the same gene. However, Defb8 was not detected by Dr Gillian Morrison working in this laboratory, and moreover the public database in the mouse genome at ENSMBL reports the peptide sequence for Defr1 and not Defb8. The solution

structure of Defb8 was elucidated in the Bauer et al. (2001) study and was found to exist only as a monomer with a similar structure to DEFB1 and DEFB4. This may suggest that the two three amino acid differences between Defr1 and Defb8 are responsible for the structural differences, although the variations in peptide synthesis may also have had an effect. Further analysis of the antimicrobial activities of all the different peptide forms (*i.e.* both monomeric Defr1 and dimeric Defr1) is required to identify any variations in their function, as the activities observed might be due to different forms of the peptide. For example, the activity seen against *B. cenocepacia* could be due to one specific form, or an interaction between several of the different forms of Defr1. DEFB103 and Defr1 have been shown to exhibit antimicrobial activity against *B. cenocepacia* and also to have salt-insensitive activity at 50 μg/ml (DEFB103 against *S. aureus* and Defr1 *P. aeruginosa*). It is interesting to note that these two β-defensins have also both been shown to exist in solution as dimers, in contrast some reports suggest that DEFB1 and DEFB4 exist as dimers only at high concentration or in crystal form (Bauer *et al.*, 2001; Schibli *et al.*, 2002). If the hypothesis suggested by Bauer *et al.* and Hoover *et al.* is correct (Hoover *et al.*, 2000; Bauer *et al.*, 2001) and β-defensins do exert their antimicrobial activity as oligomers, then the suggested ability of DEFB103 and Defr1 to oligomerise at low concentrations may explain their particularly potent activities. However, this would require further investigation to confirm if DEFB103 and Defr1 do possess the ability to dimerise at low concentrations and to investigate whether the effects of Defr1 are mediated by the monomeric or dimeric forms of the peptide. As the basis for the activity of cationic peptides is thought to be the net positive charge, it is interesting to note that DEFB103 has a very high net positive charge of +11. The net positive charge of Defr1 (+5) is higher that that of some other β-defensins, for example Defb2 (+3), DEFB1 (+4) and Defb1 (+4). It has been

suggested that the very high positive charge of DEFB103 may explain its unusual patterns of activity (Harder et al., 2001; Lehrer and Ganz, 2002b). However, whilst the charge of Defr1 may be higher that some other defensins it may not be high enough to explain the observed activities. Moreover, the net positive charge of DEFB4 is +6, even though it less potent than Defr1 against the bacteria tested. Therefore, the activity is probably dependent on more than the net positive charge. It should be noted that as the β-defensin family shows a very high rate of amino acid substitution (Hughes, 1999; Morrison *et al.*, 2002c), the observed activities could, of course, be due to any of the other amino acid changes. Further analysis of the antibacterial activities of β-defensins and in particular, the monomeric and oligomeric forms are necessary.

The importance of different individual amino acid residues in the activity of antimicrobial peptides is demonstrated by the human neutrophil peptides-(HNP-) 1, 2 and 3. HNP 1 and 2 have demonstrated potent antimicrobial activity against *Candida albicans*. However, HNP3, which differs only by an additional amino acid at the, N-terminus showed no significant activity against *C. albicans* (Lehrer *et al.*, 1988). Furthermore, in a separate study Raj *et al.*, showed that the addition of two arginine residues at both the N- and C-termini of HNP-2 significantly enhanced its antifungal and antibacterial activities (Raj *et al.*, 2000). Moreover, these modified peptides showed activity against *Actinobacillus actinomycetemcomitas* and *Porphyromonas gingivalis* whereas the native HNP-2 peptide did not. Other mutational studies have also been conducted on the α-defensins to assess the importance of structure on antimicrobial activity. A recent study synthesised various structural analogues of HNP-1, and demonstrated that cyclisation of HNP-1 lead to an increase in its potency to various Gram-negative and Gram-

positive bacteria and a reduction in its salt-sensitivity. Interestingly, it also showed that a covalent dimer formed between two cyclic HNP-1 molecules was the most potent in high salt and the only peptide to show activity against *Klebsiella pneumoniae* (Yu *et al.*, 2000). Furthermore, the recently identified θ-defensin RTD-1 shows salt-insensitivity and a 3-fold greater antimicrobial activity against *S. aureus* than the open chain analogue, which possesses an additional charge at both termini, and it is proposed that this reduces the ability of the peptide to interact with the membrane (Tang *et al.*, 1999).

3.4.7 Physiological Roles of β-Defensins

It is notable from the salt-sensitivity of the β-defensins analysed that, with a few exceptions, they do not show significant antimicrobial activity in NaCl concentrations of 150 mM, and their activity in 90 mM is greatly reduced. Elucidation of the physiological salt concentration at the sites of antimicrobial action will provide insights as to the likely endogenous relevance of these antibacterial peptides. In addition to this, the concentration of β-defensins at their site of action (e.g. the ASL of the lungs, or the surfaces of other epithelial tissues) is also of relevance here, however, there is a paucity of data regarding the concentration of the various β-defensins. One study failed to detect DEFB1 at all in broncheoalveolar lavage fluid (BALF) although the α-defensins human neutrophil peptides 1-3 (HNP1-3) were detected (Schnapp and Harris, 1998). The Singh *et al.* study previously discussed also analysed the concentration of DEFB1 and DEFB4 in the airways (Singh *et al.*, 1998). They found that DEFB1 was present in broncheoalveolar lavage fluid (BALF) at a concentration of less than 2 ng/ml, and DEFB4 was detected 0.1 –100 ng/ml. A separate study found DEFB1 and DEFB4 at low (0.05 -0.1 ng/ml) in the lungs of healthy patients and at 0.2–2 ng/ml in patients with

inflammatory lung disease (Ganz, 1998). It is thought that this is within the range of antimicrobial activity for DEFB4, but not for DEFB1 and these concentrations are well below those used in the studies reported here and much of the previously published data. The concentrations of β-defensins reported by Singh *et al.* may represent the average or overall level, whereas BALF may in reality be composed of a mosaic of high and low local concentrations. It must also be considered that the BALF is fluid from the lower airways and there may exist higher concentrations of β-defensins in the upper airways, indeed Ganz suggests as much as a 1:100 dilution of ASL in BALF (Ganz, 1998). A study of the antimicrobial peptides present in nasal secretions detected DEFB4 at concentrations ranging from 0.3 to 4 μg/ml (Cole *et al.*, 1999). Also, it could be argued that as the upper airways, such as the trachea and nasal turbinates, are likely to be where bacteria first colonise following inhalation that the level of β-defensins in this section of the airways may be of more functional importance than the lower airways such as the alveoli. Furthermore, a study analysing the concentration of α-defensins in the sputum of five different CF patients detected HNP 1-4 at concentrations of 300 to 1600 μg/ml, this concentration is well above bactericidal concentration and was sufficient to be cytotoxic to a CF tracheal epithelial cell line (Soong *et al.*, 1997). In a separate study human neutrophil peptides were detected at a concentration of up to 170 μg/ml in the plasma of patients with septicaemia (Panyutich *et al.*, 1993). In addition, the effective antimicrobial concentration has been shown to be dictated by the NaCl concentration, which as will be discussed below, remains unknown in the airways.

The complete absence of, or at least severe reduction in, the antibacterial activity of β-defensins in high levels of salt and the relatively high concentration of peptide required for the antibacterial activity observed in

the studies described in this chapter remains a confusing yet interesting finding. These observations may suggest that β-defensins are incapable of functioning as antimicrobial peptides at physiological levels of salt, indeed if their main function is as antimicrobial peptides, then why do they show least activity at physiological levels of NaCl? One explanation for this may be that their main function is not as antimicrobials, but as signalling molecules, and in fact β-defensins have been shown to act as chemotaxins reviewed in (Yang *et al.*, 2001) and to induce mast cell degranulation and regulate prostaglandin production (Niyonsaba *et al.*, 2001). It is also possible that the large number of bacteria used in this assay or the short incubation time compared to many of the other studies may artificially conceal antibacterial activity as cationic peptides are intended to kill a small number of invading bacteria not to kill a large number of bacteria. Also, as the β-defensins function at epithelial surfaces they will likely interact with other aspect of the immune system. This raises the possibility that the activity of β-defensins to act synergistically with other antimicrobial proteins, and this may serve to raise the activity of otherwise salt-inhibited β-defensins to functional levels. It has previously been shown that the HNP 1-3 can act synergistically with hydrogen peroxide to lyse tumour cells (Lichtenstein *et al.*, 1988), and DEFB4 is synergistic with either lactoferrin or lysozyme (Bals *et al.*, 1998b). Moreover, a further study showed that synergy between HNP1 and the human cathelicidin LL-37 was able to overcome the ablation of HNP1 antimicrobial activity by high levels of NaCl (Nagaoka *et al.*, 2000). It should also be borne in mind that the studies presented in this chapter used synthetic peptides and due to effects of cellular processing peptides produced *in vitro* may be more effective than those chemically synthesised. For example, Singh *et al.*, reported antimicrobial activity by DEFB4 with concentrations as low as 100 ng/ml

using recombinant peptides that had undergone cellular processing (Singh *et al.*, 1998).

3.4.8 Putative Role of β-Defensins in Cystic Fibrosis

As was discussed earlier the salt-sensitive activity of β-defensins has been implicated in the pathogenesis of CF lung disease. This theory necessarily predicts a hypotonic ASL in non-CF individuals and that loss of CFTR function leads to an isotonic ASL. This contrasts with the theory proposed by Matsui *et al.* (1998), which predicts an isotonic ASL in non-CF individuals. This theory suggests that the primary defect in CF is excessive absorption of fluid leading to dehydrated mucous and reduced mucociliary clearance (Matsui *et al.*, 1998). Consequently the concentration of NaCl in the ASL as well as the nature of β-defensin activity have become of great interest. Several studies have attempted to measure the ionic composition of ASL, however, this issue remains unresolved. In humans, the ASL is approximately 10-20 μm thick (reviewed in Pilewski and Frizzell, 1999) and consequently sampling fluid from it has proved to be technically difficult. The main technique employed has been to absorb liquid using small pieces of filter paper, however as this method generates relatively large quantities of fluid there is speculation that the collected samples do not represent normal ASL but instead secreted fluid. Studies by Joris *et al.* using the filter paper technique and Gilljam *et al.* using a dry aspiration technique found ASL to be in non-CF individuals and elevated in CF individuals (Gilljam *et al.*, 1989; Joris *et al.*, 1993). However, other studies by Zhang and Englehardt., and Hull *et al.*, using different techniques found no significant difference in ASL between CF and non-CF individuals, with Na and Cl levels between 80 and

125 mM (Hull *et al.*, 1998; Zhang and Engelhardt, 1999). Furthermore, Jayaraman *et al.* (2001) used a novel *in situ* fluorescence technique to measure the properties of the freshly secreted fluid form the submucosal glands of CF and non-CF lungs. This technique revealed the ASL pH was approximately 7 and the Na^+ and Cl^- were 92 and 94 mM respectively. Significantly, they found no differences between the salt concentration of the ASL from CF and non-CF lungs, but the fluid from CF lungs displayed great viscosity than the fluid from non-CF lungs. This important study provided evidence that rejected the hypotonic ASL/defensin theory and supported the classical view of CF pathogenesis as suggested by Matsui *et al.* (1998). Studies have also been conducted on ASL from murine airways. A capillary electrophoresis fluid collection technique was used to show that the ASL of wild-type mice is hypotonic with a Na concentration of 87 – 112 mM, but this was not found to be elevated in the *Cftr*^{tm1Unc}/*Cftr*^{tm1Unc} CF mouse model (Cowley *et al.*, 1998). Another study using the *Cftr*^{tm1Kth}/*Cftr*^{tm1Kth} CF mouse model has found evidence of hypotonic ASL with Cl levels of 18 mM, but again found no evidence for significantly elevated NaCl levels in the CF mutant mice compared to non-CF littermates (McCray, Jr. *et al.*, 1999). However, one study using a cryoprobe has found evidence for significantly elevated NaCl concentrations in the *Cftr*^{tm1Hgu}/*Cftr*^{tm1Hgu} CF mouse model compared to non-CF littermates (Zahm *et al.*, 1999). This study found very low levels of Na (12 mM) and Cl (8.5 mM) in non-CF mice; and the concentrations were elevated to 52.9 and 37.2 mM respectively in *Cftr*^{tm1Hgu}/*Cftr*^{tm1Hgu} mice. Interestingly, these mice have been demonstrated to develop lung disease following exposure to CF pathogens *S. aureus* and *B. cepacia* (Davidson *et al.*, 1995). The studies on the NaCl concentration of ASL in mice or humans have given varied results. It is interesting to note, however, that while few studies have found evidence for elevated NaCl concentrations in CF compared to non-CF

individuals (or non-CF littermates) several have reported hypotonic ASL, and consequently this is an environment in which β-defensins may be able to function as antimicrobial peptides. Clearly, further analysis of this issue is required as the concentration of NaCl is crucial to resolving the validity of the hypotonic ASL/defensin theory of CF pathogenesis. It may also be possible that the salt-sensitivity of β-defensins is not of relevance in CF pathogenesis, but dysfunction of β-defensins may still be of relevance. One further theory may be that reduced fluid movement across epithelia due to thickened mucous of CF patients may reduce the concentration of antimicrobial peptides such as the β-defensins in the ASL and thus render the airways susceptible to bacterial colonisation (Cole *et al.*, 1999).

The current studies support the salt-sensitive nature of β-defensins, and synthetic DEFB4 has potent activity against *P. aeruginosa*, which is severely reduced by 150 mM NaCl. Thus, dysfunction of DEFB4 in a high-salt environment may result in increased susceptibility to *P. aeruginosa*, and a similar trend was observed for DEFB1 against *P. aeruginosa* (Morrison *et al.*, 1998). Interpretation of the antibacterial activity for the mouse β-defensins is somewhat problematic as the bacteria analysed in this chapter are human pathogens, although both batches of Defb2 did show a significant reduction in the antibacterial activity against *P. aeruginosa* in the presence of salt. Defr1 has highly potent, and to some extent salt-insensitive, activity against *P. aeruginosa*, however, Defr1 expression was not detected in the airways (Morrison *et al.*, 2002a). It is possible therefore, that alternative antimicrobial peptides or cellular components of the immune system may mediate resistance to *P. aeruginosa* in the airways and these may not be impaired by high salt concentrations. The evolution of species-specific spectrums of activity within the β-defensins suggests that the dysfunction of β-defensins

such as Defb2 in high levels of salt may increase the susceptibility of mice to different, mouse-specific spectra of bacteria. This might suggest that whilst the underlying defect remains the same in mice and humans, the effect of that defect is to make the organism susceptible to different spectra of bacteria. Further, analysis of the spectrum of mouse pathogens needs to be examined.

3.4.9 Summary

In conclusion, this chapter has presented data that demonstrates that synthetic β-defensins DEFB4, Defb2 and Defr1 are salt-sensitive antibacterial peptides. In the absence of salt human DEFB4 kills *P. aeruginosa* J1385 and PAO1 very efficiently, *S. aureus* C1705 was also killed quite efficiently, but less so than *P. aeruginosa*. The mouse β-defensin, Defb2, also killed *S. aureus*, but there were conflicting results regarding the activity of Defb2 against *P. aeruginosa*. The first batch showed only mild activity, but the second batch demonstrated significantly greater activity, giving a profile more similar to that seen with DEFB4. Neither DEFB4 nor Defb2 killed *E. coli* J2408 or *B. cenocepacia* J2315, but weak antimicrobial activity was seen against *E. coli* J3201. The antimicrobial activities, however, were ablated by high levels of NaCl, and this may contribute to species-specific susceptibility to bacterial infection secondary to CFTR dysfunction. Studies with synthetic Defr1 showed that this peptide is a highly potent antimicrobial peptide, which kills *S. aureus* C1705 and *E. coli* J2408 highly efficiently in the absence of salt, but this activity was also ablated by high levels of salt. However, its activity against *P. aeruginosa* was salt insensitive and it maintained a high degree of potency even at comparatively low concentrations. Surprisingly, Defr1 also

showed antimicrobial activity against B. cenocepacia, the first β-defensins to demonstrate such activity.

These studies provide highly interesting results regarding the activity of human and mouse β-defensins. However, more studies of the activity of other members of the β-defensin family as well as mutational studies are required to further elucidate the structure-function relationships of this family of antimicrobial peptides. Greater analysis of the role of β-defensins in the ASL of the airways and at other epithelial surfaces is also desirable to allow better understanding of the role of β-defensins in the innate immune system.

4.1.1 Introduction

Chemotaxis is defined as a process of directed migration of cells along a chemical gradient. This process is recognised as an important cellular process and has been shown to be important in cancer metastasis, embryogenesis and the inflammatory response. One of the main areas of study of chemotaxis has been its role in the immune system where large number of chemoattractant substances have been identified of which one of the main families is the chemokines. Chemokines are low molecular weight chemoattractant cytokines, they induce extravasation, chemotaxis and activation of a wide variety of leukocytes and other immune cells for review see (McFadden and Kelvin, 1997). However, both human α- and β-defensins, and other cationic antimicrobial peptides such as the cathelicidin LL-37, have also been shown to possess chemoattractant properties towards neutrophils, monocytes, T-lymphocytes, dendritic cells and mast cells reviewed in (Yang *et al.*, 2001). The defensin-induced migration of T-lymphocytes is particularly interesting as they were first recognised as antibacterial peptides, and such a mechanism provides a link between the innate and adaptive immune systems. However, chemoattractive ability is not a universal property of defensins as Guinea pig neutrophil defensins GNCP1 and 2 do not induce migration in neutrophils, whereas human neutrophils peptides HNP 1 and 2 induced migration in neutrophils (Chertov *et al.*, 1996). Moreover, whilst studies have shown HNP1 and 2 to be chemoattractants for monocytes, CD4+ and CD8+ T-lymphocytes and immature dendritic cells, HNP3 has not been found to induce migration of these cells (Territo *et al.*, 1989; Yang *et al.*, 2000a). To date induction of cellular migration has not been reported for

murine β-defensins, with the exception of a study by Biragyn *et al.*, which showed that murine β-defensins fused to the lymphoma antigen sFv can induce migration of dendritic cells (Biragyn *et al.*, 2001). This study demonstrated that Defb2 or Defb3 peptides fused to the sFv antigen induced migration of immature, but not mature, dendritic cells, whereas the sFv antigen alone did not induce migration. However, the authors did not analyse the migration induced by the β-defensins alone.

This chapter presents data analysing the chemoattractant activity of the synthetic murine β-defensins, Defr1 and the second batch of Defb2 that were shown in the previous chapter to possess antimicrobial activity. The studies were performed using a 48-well microchemotaxis chamber (Falk *et al.*, 1980) and the ability of different concentrations of β-defensins to induce migration in neutrophils, CD4+ T-lymphocytes and dendritic cells was analysed. Neutrophils were isolated from mouse peritoneum following the method of Rot (1991). CD4+ T-lymphocytes were isolated form mouse spleens and purified by selecting for CD4-expressing cells using the autoMACS system (Miltenyi Biotec). Dendritic cells (DCs) were derived from bone marrow cells cultured in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) (Inaba *et al.*, 1992). Previous reports have shown that α- and β-defensins attract immature but not mature dendritic cells (Yang *et al.*, 1999; Yang *et al.*, 2000a). Therefore, the chemoattractant abilities of Defb2 and Defr1 were analysed against immature DCs that had been cultured for 6 days (d6iDC), immature dendritic cells cultured for 7-days (d7iDC) and mature dendritic cells (mDC).

4.1.2 Chemotaxis of Immune-Related Cells

Many immune factors have chemotactic activity such as the group of chemotactic cytokines (called chemokines), some complement factors (e.g. C5a) and, as discussed above, some antimicrobial peptides (e.g. α- and β-defensins and LL37) (reviewed in Yang *et al.*, 2001 and in Mackay, 2001). Most types of immune-related cells possess the ability for chemotaxis (reviewed in Luster, 2002). In chapter 4 of this thesis, the migration of neutrophils, T-cells and dendritic cells in response to murine β-defensins is analysed and what follows is a brief introduction to these cell types.

4.1.3 Migration towards sites of Infection

Many immune cells will migrate or 'patrol' to some degree throughout the body and upon stimulation with inflammatory cytokines will migrate more specifically to the site of infection by following chemotactic gradients (reviewed in Delves and Roitt, 2000a; Delves and Roitt, 2000b). Inflammatory signals, such as LPS, induce the up-regulation of adhesion molecules such as the E-selectins on the surface on the vascular endothelium. The E-selectins on the endothelium bind sialylated carbohydrate ligands, often displayed mucin-like molecules (Springer, 1994). These interactions are weak and cause the patrolling cell to 'roll' along the endothelium (Figure 4.1A). This is followed by rapid activation of integrins on the leukocytes. These interact with immunoglobulin superfamily (IgSF) members, such as ICAM1 and 2, expressed on the endothelial surface (Figure 4.1B). The main function of this interaction is to arrest the rolling process and to bring about firm adhesion to the endothelial surface (Figure 4.1C). The activated neutrophil then passes through the endothelial wall (Figure 4.1D), moving

up the chemotactic gradient to accumulate at the site of infection where it can function as an effector cell (Figure 4.1E, F).

The immune cell is attracted through the endothelium by interaction with various chemokines that are immobilised on the endothelial surface by presentation molecules such as proteoglycan and possibly also due to direct production of chemokines by endothelial cells (reviewed by Witko-Sarsat *et al.*, 2000). It has been suggested that the cell migrates through the endothelium via interaction between immune cell expressed integrins and P-selectins, which have been shown to be concentrated on endothelial cell borders (Burns *et al.*, 2000). Homophilic interactions between the cell adhesion molecule CD31, which is expressed on the surface of both leukocytes and endothelial cells, are also important for transendothelial migration, and a zipper model has been proposed to account for maintenance of endothelial permeability (Muller *et al.*, 1993). It has been suggested that leukocytes navigate through complex gradients of chemoattractants in a stepwise fashion by responding to one agonist source after another. The direction of leukocytes movement is directed by first the steepest local chemoattractant gradient and is then regulated by successive receptor desensitisation and attraction by distant agonists and finally, end-target attractants (e.g. formyl peptides, C5a) are dominant over regulatory cell derived agonists (e.g. interleukin-8, leukotriene B_4) (Witko-Sarsat *et al.*, 2000 and Foxmann *et al.*, 1999). Chemotactic cells are typically elongated and more sensitive to an attractant at one end. Chemoattractants are detected by G-protein-coupled receptors (Iijima and Devreotes, 2002). However, previous studies have shown that the chemotactic receptors are uniformly distributed on the cell surface and therefore it is proposed that the signalling pathways must be selectively activated on the cells leading edge (Servant *et*

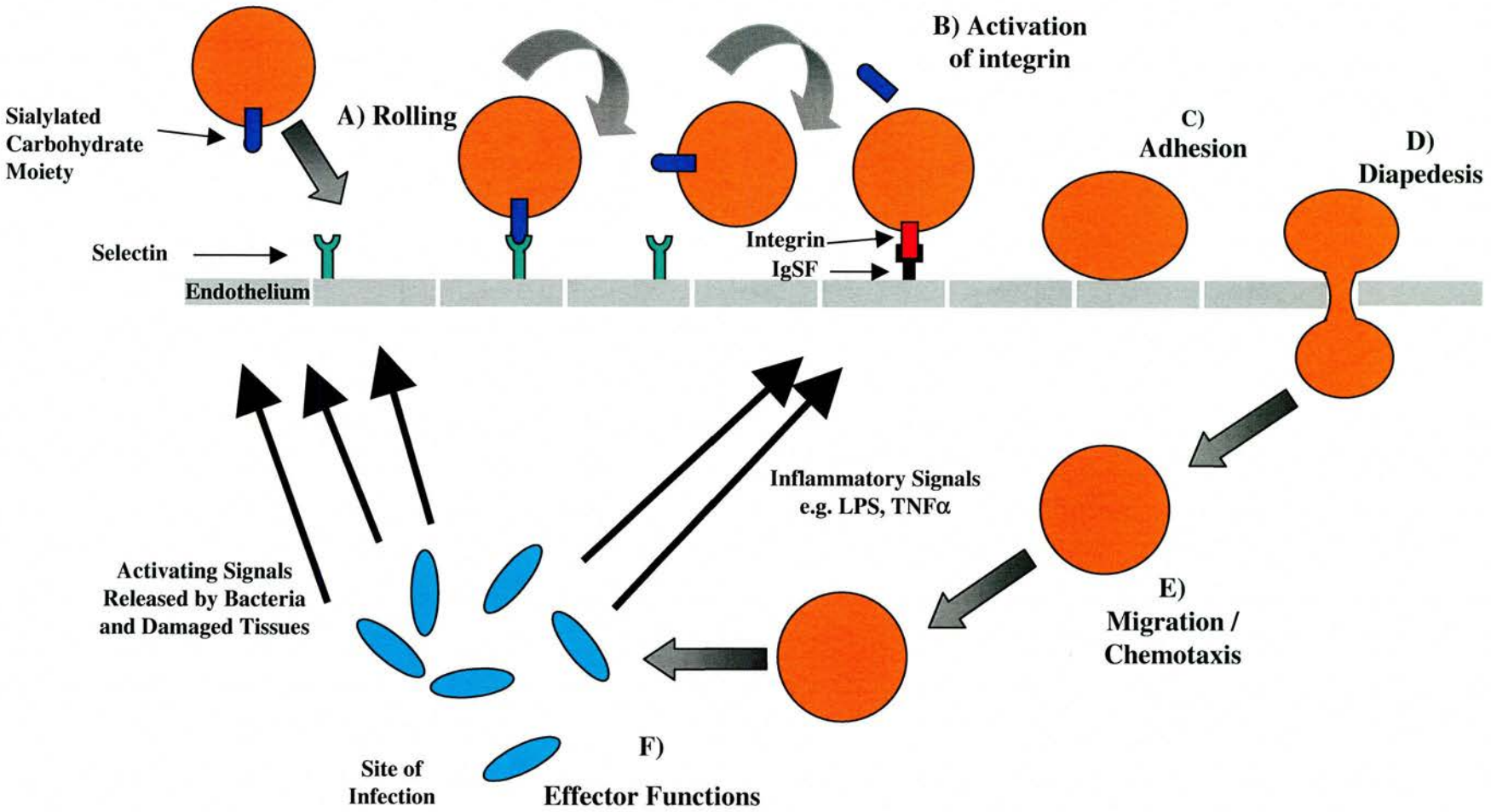

Figure 4.1: Migration of Immune Related Cells to Site of Infection. Inflammatory stimuli induce the expression of selectins on the endothelial surface. These interact with carbohydrate ligands, often displayed on mucin-like molecules, which are expressed by the leukocytes and cause the cell to 'tether' to and roll along the endothelial surface. This is followed by activation of integrins on the leukocyte. Integrins interact more strongly with immunoglobulin superfamily (IgSF) members, such as ICAM-1, expressed by the endothelium, causing the leukocyte to cease rolling and adhere to the endothelium. This process is followed by transmigration through the endothelium and migration to the inflamed site.

al., 2000). The mechanism by which the cell senses the direction of the gradient remains unknown, however one theory proposed to explain this is the local excitation/global inhibition theory (Parent and Devreotes, 1999). This theory is based upon a balance between rapid excitatory and slower inhibitory processes, which are each controlled by receptor binding, regulating the response. When the receptor is bound by a chemoattractant, the excitation signals rapidly increase and a response is generated until inhibition signals reach the same levels. In a chemical gradient the excitation signals at the front of the cell exceeds the inhibitory signals at the back, which generates a persistent excitatory signal, and moreover at the back of the cell the inhibitory signal predominates and therefore there is no response. Thus, according to this model, the duration of the response is related to the cells localisation within a chemical gradient. The signal results in localised polymerisation of F actin at the site of the cell cortex closest to the chemotactic source, leading to the formation of a new pseudopod, cell polarisation and the forward protrusion of the leading edge. This is followed by assembly of conventional nonmuscle myosin and retraction of the posterior of the cell (Parent and Devreotes, 1999).

4.1.4 Neutrophils

Neutrophils are among the first immune cells to arrive at the site of infection and are important contributors to the acute inflammatory response (reviewed in Witko-Sarsat *et al.*, 2000). Neutrophils are activated by cytokines produced primarily by macrophages and endothelial cells and their main role is to migrate to sites of infection (as described above) and into the infected site, where they phagocytose foreign particles and release the contents of the different granules that they contain. Neutrophils also possess

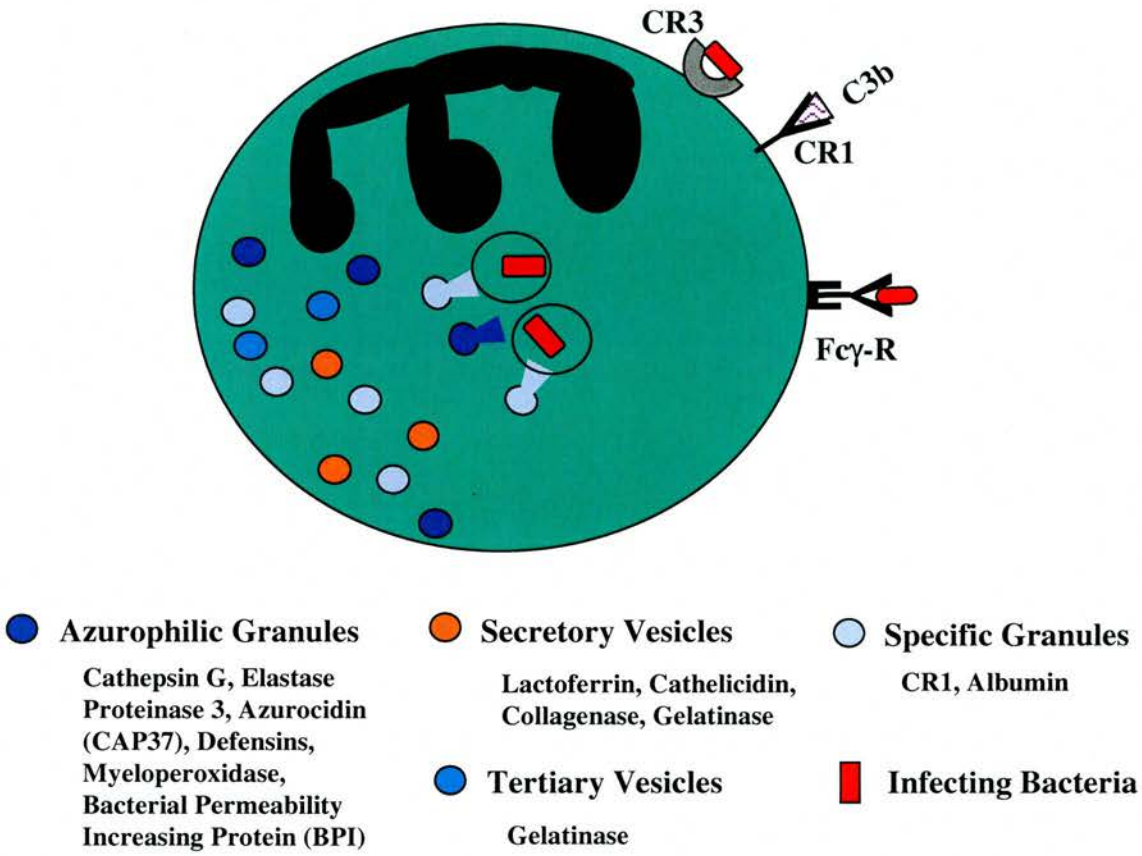

Figure 4.2: Neutrophil Effector Functions. The complement opsonins C3b and and C4b are recognised by CR1 and CR3, whereas IgG opsonised particles are recognised via the immunoglobulin receptors (Fcγ-R). This leads to activation of the neutrophil. The first microbicidal pathway is the oxidative response, which consists of the production of radical oxygen species following NADPH-oxidase complex activation, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and via myeloperoxidase, hypochlorous acid (HOCl) and chloramines. The second microbicidal pathway is non-oxygen-dependent and consists of the release in the phagolysosome or in the extracellular medium of performed proteins stored in granules. Serine proteases, antimicrobial proteins, as well as myeloperoxidase are contained in the in azurophilic granules. Metalloproteinases such as collagenase and gelatinase, and antimicrobial peptides such as lactoferrin and cathelicidin are contained in specific granules. Gelatinase is also contained in tertiary granules. Figure taken from Witko-Sarsat *et al.* (2000).

receptors for the antibody IgG (Fcg-R) and complement proteins (CR) and they migrate to and accumulate at sites of complement activation; therefore, they actively phagocytose opsonised particles and function as effector cells of humoral immunity. The various effector functions of neutrophils are summarised in Figure 4.2.

The azurophilic granules of neutrophils contain α-defensins, which have been shown to be chemotactic for immature dendritic cells, and T-cells, but not neutrophils (Yang *et al.*, 2000a), moreover studies using β-defensins have shown that they do not induce migration of neutrophils. However, cathelicidins, which are contained within the specific granules of neutrophils, do show chemotactic activity for neutrophils, suggesting the existence of a peptide-mediated positive feedback mechanism for neutrophil recruitment at the site of infection (Yang *et al.*, 2000b). Data analysing the migration of neutrophils in response to the murine β-defensins Defb2 and Defr1 are presented in chapter 4 of this thesis.

4.1.5 $CD4^+$ T-Lymphocytes

CD4+ helper T-cells are lymphocytes that secrete various cytokines required for the functional activity of other cells in the immune system. At least two types of helper T-cells are recognised. Type 1 helper (Th1) T-cells secrete the cytokine interferon-γ, but not IL-4, -5, or –6 and are mainly involved in activation of macrophages and cytotoxic $CD8^+$ T-cells. In contrast, type 2 helper (Th2) T-cells produce IL-4, -5 and –6 (but not IFN-γ), their main function is in humoral immunity, each CD4+ T-cell subtype inhibits the activity of the alternate type (Delves and Roitt, 2000a; Delves and Roitt, 2000b). Most T-cell responses are initiated by antigen presenting cells (APCs)

in the T-cell dependent areas of the lymphoid tissues, through local release of IL-12. These APCs skew the naïve T-cells towards production of either the Th1 or Th2 subset of cytokines (reviewed in Sprent and Surh, 2001).

Cytotoxic (CD8-expressing) T-cells kill cells infected with intracellular pathogens and thus interact with MHC I molecules that are expressed on the surfaces of most cells. CD4+ T-cells, however, are mainly cytokine secreting cells and respond primarily to extracellular pathogens. Antigens generated form such pathogens are presented on MHC II molecules by APCs; three cell types are recognised as APCs: dendritic cells (see below), B-lymphocytes and macrophages (reviewed in Thery and Amigorena, 2001). Interaction between an antigen-primed APC and a CD4+ T-cell can lead to its activation and the subsequent production of cytokines and activation of appropriate immune responses.

As indicated above, Th1 CD4 T-cells activate cytotoxic CD8+ T-cells and macrophages (Figure 4.3A). Upon interaction with an antigen/MHC II complex expressed on the surfaces of macrophages they produce IFN-γ by the Th 2 cells activates CD8 T-cells and macrophages to kill intracellular pathogens and IFN-γ also sets up a state of cellular resistance to viral infection (reviewed in von Andrian and MacKay, 2000).

Antigens are taken up by B-cells functioning in their APC role, processed and then presented at the cell surface on MHC II molecules. Neighbouring Th2 CD4+ T-cells that recognise these peptide-MHC II complexes become activated and express costimulatory molecules such as CD154 (also known as CD40 ligand) on their surface; interaction of CD154 with the CD40 expressed on B-cells prompts the B-cells to begin the process of somatic hypermutation

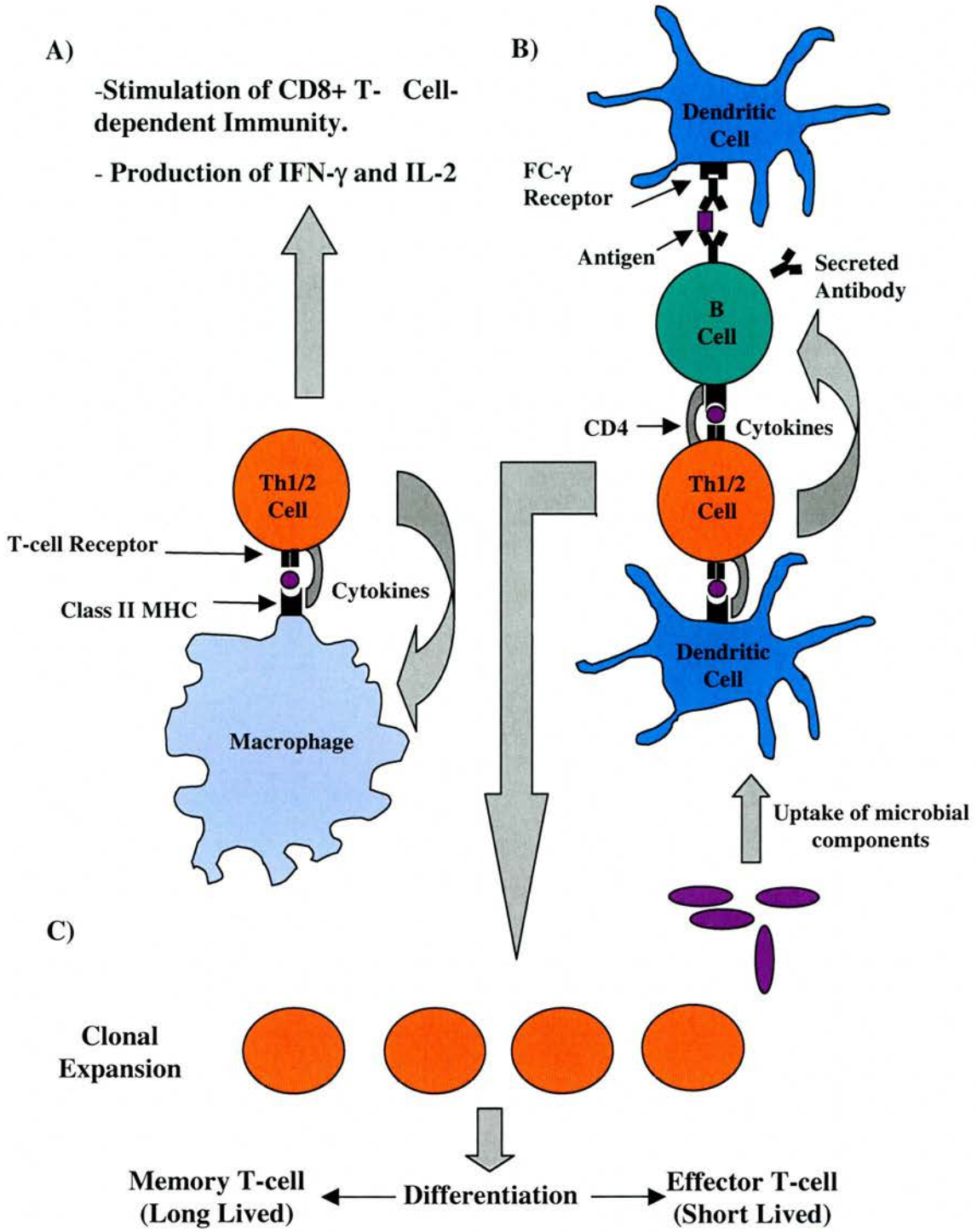

Figure 4.3: T-Cell Responses and Generation of Memory Cells. $CD4^+$ T-cells can stimulate cytotoxic T-cell responses (A), as well as humoral responses. Most of these reactions occur in the T-cell areas of the lymphoid organs following interaction with dendritic cells (B). Bacterial products are taken up and presented to T-helper cells by dendritic cells or B-cells acting in their antigen-presenting role. This leads to activation of the T-helper cells. Following activation T-cells undergo clonal expansion and differentiation into effector cells and memory cells (C). Figure adapted from Delves and Roitt (2000b) and von Adrian and Mackay (2000).

and immunoglobulin class switching (Figure 4.3B). Moreover, this process is aided by the production of IL-4, -5 and –6, which are also secreted by the $CD4^+$ T-cell.

A hallmark of the immune system is that the responses to a second infection by a pathogen are generally manifested more quickly and are more vigorously than the initial response. This immunological memory is present at the level of the T-cell and the B-cell. T-cell activation is followed by clonal expansion and differentiation, and a proportion of naïve (i.e. T-cells that have not yet been exposed to antigen and subsequently activated) T-cells differentiate into short-lived effector cells and long-lived memory T-cells following this initial antigenic challenge (Figure 4.3C) (Sprent and Surh, 2001). Thus, memory T-cells are distinguished from naïve T-cells in that they are hyper-responsive to antigen and in synthesizing cytokines in large quantities, and in particular whether they secrete the Th1 or Th2 subset of cytokines. However, the mechanisms behind the generation of immunity (*i.e.* why do some T-cells cells survive?) are unknown, although it has been proposed that the capacity of some T-cells to evade death is based on the CD40 ligand on the surface of the T-cell with CD40 on the surface of the APC. In the absence of the CD40L-CD40 interaction, there is only a limited generation of memory cells, however, other costimulatory molecules on APCs, such as B7, have all been shown to play a role in generation of memory cells (Sprent and Surh, 2001).

Interestingly, it has been shown that α- and β-defensins elicit migratory responses from different T-cell subsets, α-defensins such as HNP1 and 2 induce migration of naïve CD4+ T-cells, whereas DEFB2 induces migration of the memory subset of $CD4^+$ T-cells (Yang *et al.*, 1999; Yang *et al.*, 2000a).

CD8+ T-cells have been shown to migrate in response to α-defensins, it is not known if they respond to β-defensins or if a similar subset-specific activity exists (Yang *et al.*, 2000a). The reason for this difference in response is unclear, but may be associated with the different sites of expression of α- and β-defensins and different expression of chemokine receptors. Data on the migration of murine $CD4^+$ T-cells in response to murine β-defensins is presented in chapter 4 of this thesis.

4.1.6 Dendritic Cells

Dendritic cells (DCs) are the main professional antigen-presenting cells in the immune system and data analysing the migration of DCs in response to murine β-defensins is presented in chapter 4 of this thesis. Like T-cells, generally exist in a 'resting' phase in the absence of challenge, but are rapidly activated upon contact with an inflammatory stimulus (Delves and Roitt, 2000a; Delves and Roitt, 2000b). DCs are distributed throughout the tissues of the body where they act as 'sentinels' and constantly sample the surrounding environment. Upon detection of an antigen, DCs become activated and migrate to the lymphoid organs, which is the main site for T-cell activation. DCs generate three signals in the activation process. Signal 1 is the delivery of antigen to T-cells on MHC II molecules, signal 2 is the up-regulation of costimulatory molecules, with a consequent increase in immunogenicity to T-cells and signal 3 is the production of cytokines such as IL-10, 12 or 18 which can direct subsequent T-cell differentiation (reviewed in Reis e Sousa, 2001).

DCs are directly activated by contact with pathogen-associated molecular patterns (PAMPs) with a pattern recognition receptor (PRRs). PAMPs are

evolutionarily conserved molecules, essential to pathogen function, which are absent from the host such as lipopolysaccharide or CpG motifs of bacteria or β-glucans in yeast and other fungi. These molecules have been exploited by the host in the development of PRRs such as toll-like receptors (TLR) to detect their presence and thus mount appropriate immune responses.

DCs also exist in two different states – immature and mature, the two types are distinguished by the organisation of antigen-presenting capacity and their function (reviewed in Watts, 1997). Immature DCs show high rates of endocytosis and antigen processing, however they do not efficiently present antigen on the cell surface. In contrast, mature DCs show reduced levels of endocytosis, but elevated rates of antigen presentation. Thus, in an immature state – prior to exposure to inflammatory stimuli – DCs act as 'sentinels', readily sample, and process the local milieu. In this state the DC MHC II biosynthesis, endocytosis and subsequent processing of the ingested products to MHC II-peptide complexes occurs, however these are largely maintained in the lysosomes or reach the cell surface and are then rapidly recycled (Figure 4.4A). The maturation process is initiated upon stimulation with inflammatory stimuli such as TNFα or LPS and the DC then changes function from sentinel to antigen presenter; there is increased biosynthesis of MHC II, which maximises loading of antigen onto the MHC II molecules (Figure 4.4B). The DC then migrates from the non-lymphoid tissue, where the stimulation occurred to the T-cell rich areas of the lymphoid organs. Following migration, the MHC-II/peptide complexes are efficiently expressed on the surface of the cell, where they persist for longer periods than in the immature DCs, due to the reduction in endocytosis (Figure 4.4C).

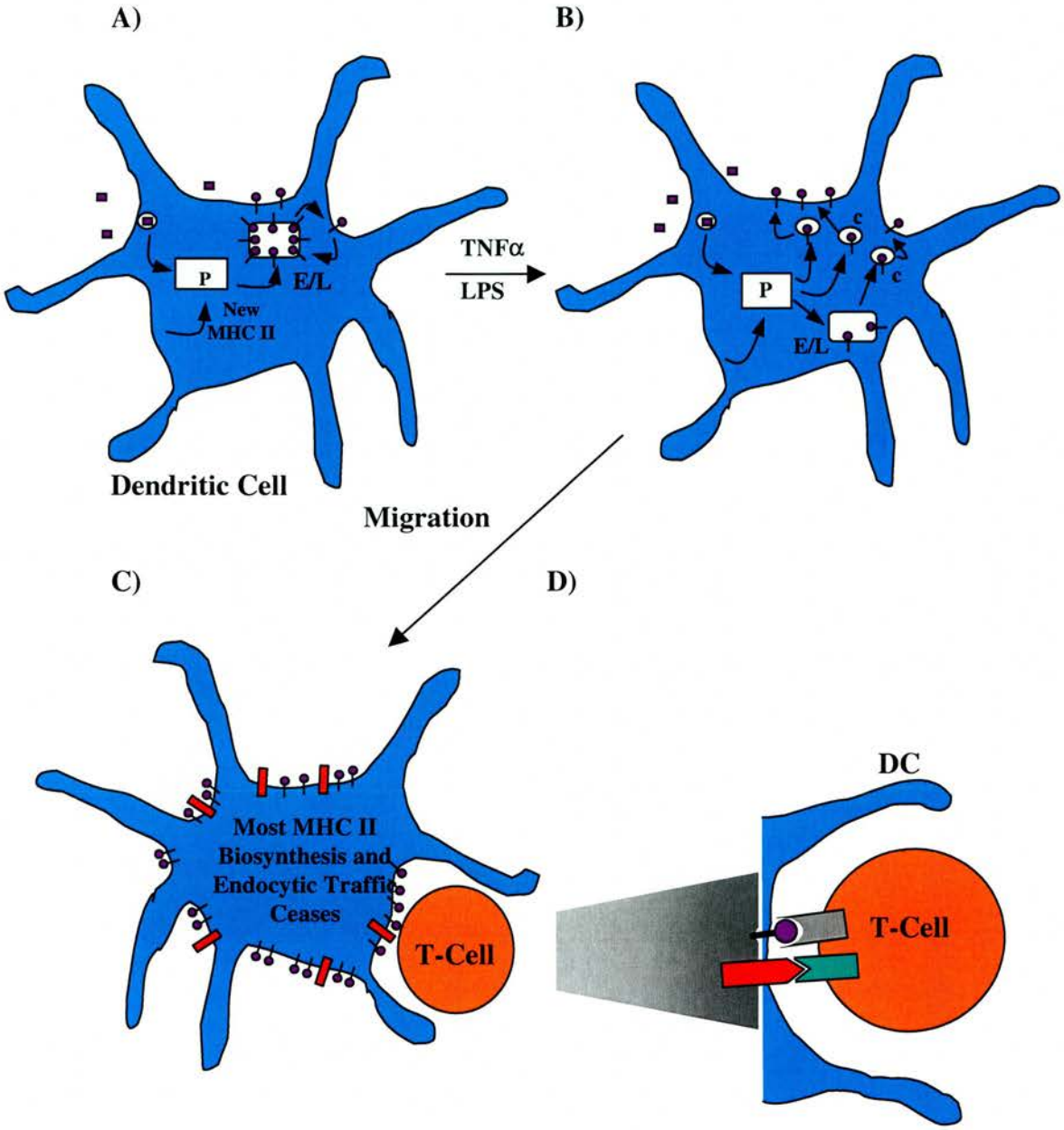

Figure 4.4: Dendritic Cells act as Sentinels in the Non-Lymphoid Tissues and, Following Stimulation by Inflammatory Stimuli, Migrate to the Lymphoid Organs where they act as Antigen Presenting Cells.

A) Endocytosis (purple squares) and MHC biosynthesis occurs but the assembled complexes (purple circles and black sticks) accumulate in the lysosomes of mouse DCs (L) or the endosomes of human DCs (E) or reach the cell surface and are then recycled. **B**) Following stimulation by inflammatory stimuli the DC starts to mature. There is increased synthesis of MHC II and elevated loading in the peptide-loading compartments (P), CIIV (c) and recycling endosomes. **C**) In the mature DC the peptide/MHC II complexes are highly expressed on the cell surface to permit activation of the T-cells. This occurs following DC migration to lymphoid organs. **D**) T-cells are activated by interaction of MHC II-peptides complexes with T-cell receptor (illustrated in grey with crescent shaped binding site) and this activation is greatly amplified by interaction of B7 molecules (shown in red) with CD28 (shown in green) on the surface of the T-cell. Figure Adapted from Watts (1997) and Delves and Roitt (2000).

Moreover, there is also increased expression of costimulatory molecules (e.g. CD40, CD80) on the cell surface, which are required for the stimulation of T-cells (reviewed in Banchereau and Steinman, 1998). It is known that DC populations produce different cytokines in response to different activating stimuli. However, DC populations are heterogeneous; and it is unclear if the different types of immunity are generated by different DC subtypes responding to different PAMPs (*i.e.* DC1 and DC2 stimulate Th1 and Th2 T-cell responses respectively), or if a single DC type delivers distinct signals (*i.e.* different costimulatory molecules) depending on the activating stimulus (reviewed in Moser and Murphy, 2000).

4.1.7 Measuring Cell Migration

The microchemotaxis chamber designed by Falk *et al.* (1980) used in this study allows for rapid assessment of cell migration in multiple wells. The chamber consists of upper and lower chamber separated by a membrane filter of defined pore size. The chemoattractive compound is placed in the lower chamber, cells are added to the top chamber and following a period of incubation, cells that have migrated towards the chemoattractant are counted on the lower surface of the filter. Neutrophil migration was assessed after 1 hr incubation, migration of CD4+ cells was assessed after 4.5 hrs and DCs were analysed following incubation of period of 1.5 hrs. Following the appropriate incubation period the migration $\approx 1 \times 10^5$ cells in response to 0.1, 1 10, 100, 1000 and 10000 ng/ml of peptide was assessed. Cell migration was assessed by counting the number of cells on the lower side of the filter in three random fields of view (FOV) from each of the three replicate wells. Experiments were repeated a minimum of three times to ensure reproducibility and representative experiments are indicated below.

4.2 Chemoattractant Activity of synthetic murine β-defensins Defb2 and Defr1

Using a 48-well microchemotaxis chamber, the murine β-defensins Defb2 and Defr1 were tested for their chemotactic properties. The cell types assessed for chemoattractant activity were peritoneal neutrophils, CD4+ splenic T-lymphocytes, and immature and mature bone marrow-derived dendritic cells. Peptides were diluted in chemotactic media (RPMI 1640 + 10% FCS) a concentrations of 0.1 to 10000 ng/ml. Cells were isolated as described and also resuspended in chemotactic media at a concentration of $5x10^6$ cells/ml. Filters were scored by counting the number of stained cells in random field of view (FOV). Migration was assessed in three replicates wells and three FOVs were counted per well. Data is presented both as the mean number of cells per FOV and also as a migratory index (MI). This represents the fold-increase of migration by expressing the number of migrated cells per test sample as a ratio of the number of migrated cells from the control wells (media alone). Studies were tested for reproducibility in a minimum of three experiments and representative experiments are illustrated below in Figures 4.1 – 4.12.

4.2.1 Chemoattractant Activity of Defb2 and Defr1 to Neutrophils.

Neutrophils were isolated from the peritoneum and incubated with the peptide for 1.5 hrs as previously in the materials and methods, filters were then stained with DiffQuick. Microscopic analysis of the isolated cells showed that they possessed the typical neutrophil polymorphonuclear structure. The neutrophils showed significant migration in response to 10^{-7} M

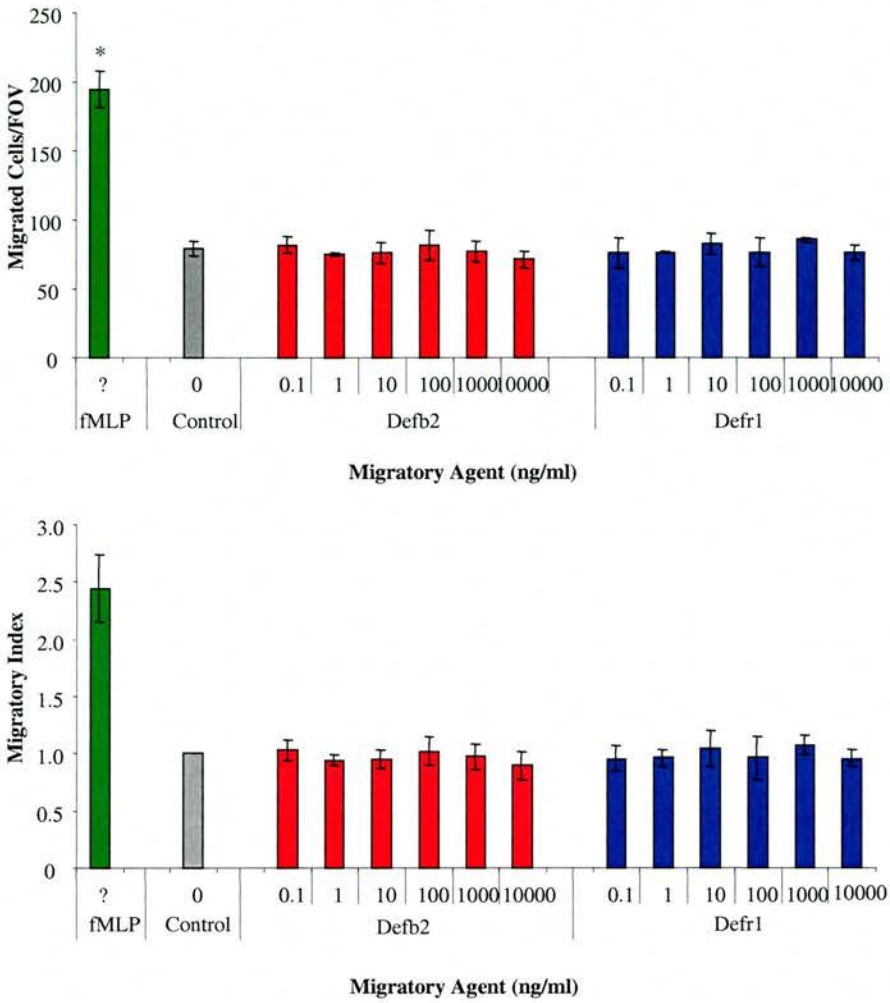

Figure 4.5: Migration of neutrophils in response to the mouse β-defensins Defb2 and Defr1.

Neutrophils were isolated from mouse peritoneum. Cells were incubated in a 48-well chemotaxis chamber for 4.5 hours separated from different concentrations of Defb2 or Defr1 by a 5μm polycarbonate filter. fMLP was used a positive control. The filter was stained with DiffQuick and three fields of view were counted in each of three replicate wells. Graph A shows mean number of cells migrated per field of view form a total of nine random fields. Graph B shows the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the control (media alone) sample. * represents $p< 0.05$. Error bars represent the standard deviation of all nine replicate counts. Experiments were checked for reproducibility in a minimum of three experiments (*i.e.* n=3) , and a typical result is illustrated above.

of the positive control, *N*-formylmethionyl leucyl phenyalanine (fMLP). The background level of migration was quite high with an average of ≈80 cells having migrated per FOV, but in response fMLP induced a significant increase in migration ($p < 0.05$). An average of ≈180 cell migrated in response to fMLP, giving a MI of almost 2.5 (Figure 4.5). However, no migration was observed in response to Defb2 or Defr1 above the background level. This suggests that synthetic Defb2 and Defr1 do not induce cellular migration in neutrophils. Isolated cells were confirmed as neutrophils by morphology and all populations used were at least 90% pure.

4.2.2 Chemoattractant Activity of Defb2 and Defr1 to splenetic $CD4^+$ T-lymphocytes.

$CD4^+$ T-lymphocytes were isolated from mouse spleens by the magnetic cell sorting (MACS) as described in the materials and methods and incubated with the peptides for 4.5 hours after which filters were stained with haematoxylin and scored. In the control (media alone) sample there was a low level of migration with approximately 30 cells per FOV (Figure 4.6). In the presence of 10 ng/ml of the positive control (stromal derived factor-1alpha (SDF1α)), the MI was 4.5, with an average of 135 cells per FOV. CD4+ T-cells showed significant migration in response to Defb2 and Defr1 ($p < 0.05$). The migration induced was concentration-dependent and peaked at 10ng/ml with ≈145 cells/FOV (MI of 5.0). A concentration of 1 ng/ml of Defb2 induced a 4-fold increase in migration, with ≈120 cell/FOV. A concentration of 0.1, 100 or 1000 ng/ml induced 2.5- 3–fold increase in migration (approximately 75-90 cell/FOV), and at 10000 ng/ml the MI was less than 2.0. Concentration-dependent migration was also observed

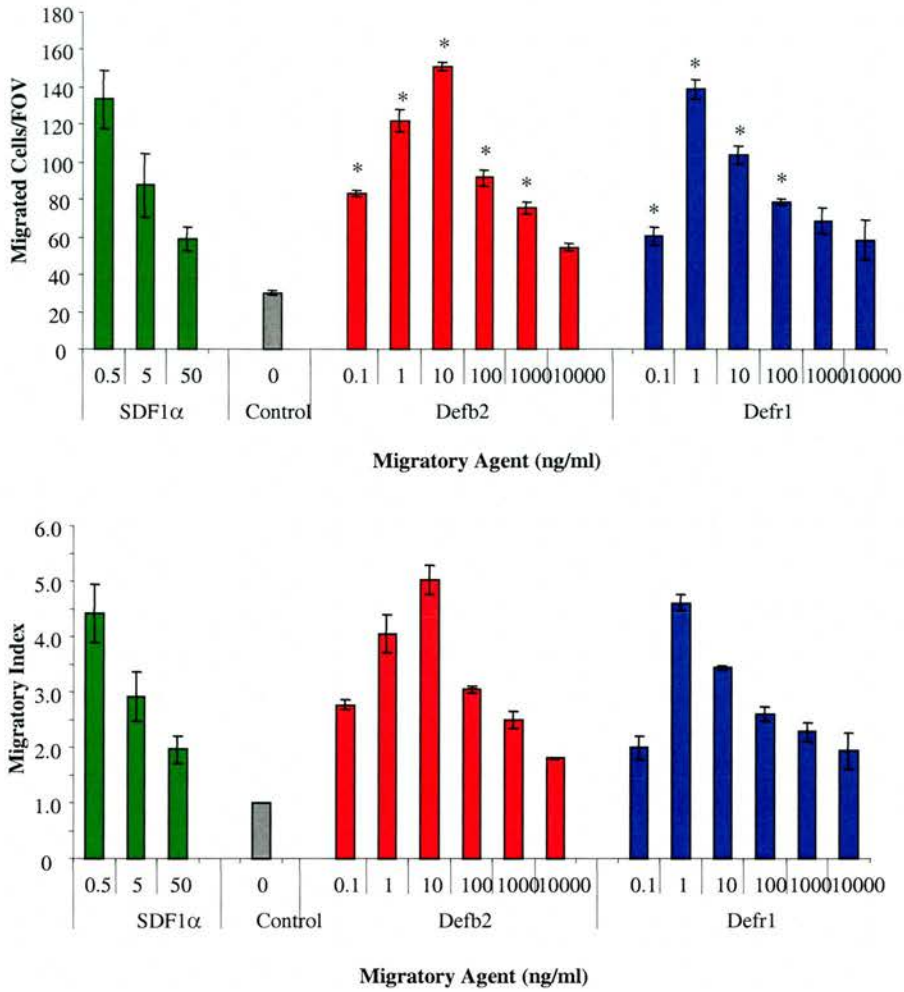

Figure 4.6: Migration of CD4+ T-lymphocytes in response to the mouse β-defensins Defb2 and Defr1.

CD4+ T-lymphocytes were isolated from mouse spleens by autoMACs separation. Cells were incubated in a 48-well chemotaxis chamber for 4.5 hours separated from different concentrations of Defb2 or Defr1 by a 5μm polycarbonate filter. SDF1α was used a positive control. The filter was stained and three fields of view were counted in each of three replicate wells. Graph A shows mean number of cells migrated per field of view form a total of nine random fields. Graph B shows the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the control (media alone) sample. * represents $p< 0.05$. Error bars represent the standard deviation of all nine replicate counts. Experiments were checked for reproducibility in a minimum of three experiments (*i.e.* n=3) , and a typical result is illustrated above.

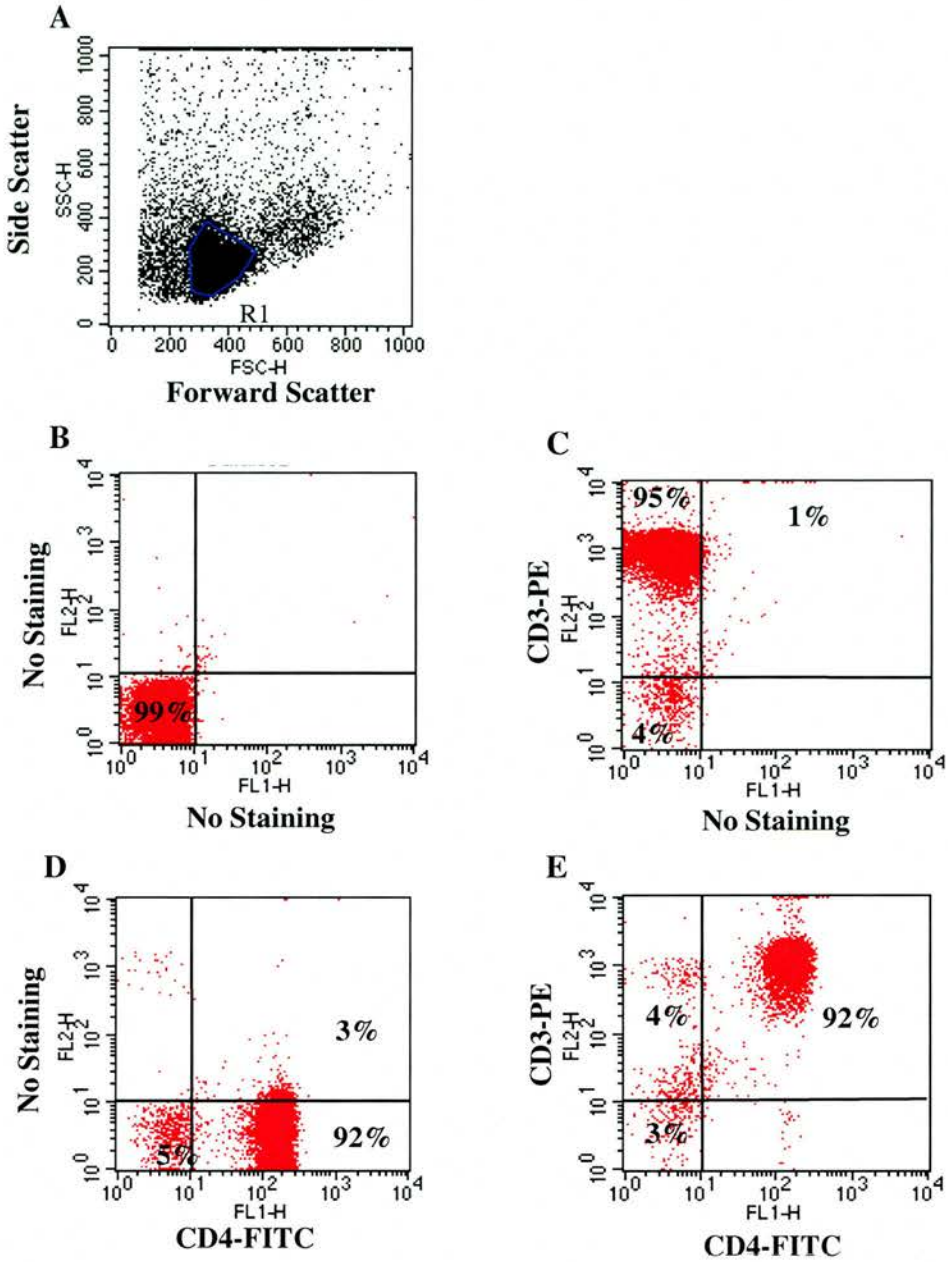

Figure 4.7: FACS analysis of CD4⁺ T-lymphocyte surface antigen expression.

CD4+ T-lymphocytes, isolated from mouse spleen using the autoMACS system (Milteny Biotec), were double stained for CD3 and CD4 expression. A) shows forward and side scatter and R1 gate used in subsequent analysis. B) Dot plot of unstained cells. C) shows a dot plot of cells stained for expression of CD3. D) show a dot plot of cells stained for CD4. E) shows cells double-stained for CD3 and CD4 expression. All antibodies were either PE- or FITC-conjugated as indicated. Quadrant grid was set using isotype controls. FACs analysis was performed on all three replicate experiments to ensure reproducibility of the isolated cells phenotype (n=3).

in response to Defr1, the peak level of migration was seen at 1 ng/ml a lower concentration than that observed for Defb2. At 1 ng/ml the MI was 4.5 with an average of ≈140 cell/FOV, whereas at 10 ng/ml a 3.5-fold increase in migration was observed compared to the control wells. The migration induced by 10 ng/ml Defr1 was statistically significantly lower than that induced by the same concentration of Defb2; there were no differences between Defb2 and Defr1 at other concentrations tested. At a concentration of 100 or 1000 ng/ml migration of approximately 70 cells/FOV was observed, giving a MI of 2-2.5. Much lower migration was observed with 0.1 or 10000 ng/ml with a MI of only 1.5. The migration induced by Defr1 at 1000 or 10000 ng/ml was not statistically significant compared to control.

Isolated cells were analysed for expression of the cell surface markers CD3 and CD4 by fluorescence-activated cell sorting (FACS). Analyses showed that 92% of cell were double-positives and expressed both CD3 and CD4 (Figure 4.7). This confirms the purity of the isolated cells as CD4+ T-lymphocytes.

	d6iDC		d7iDC		mDC	
Surface Antigen	**%**	**mean**	**%**	**mean**	**%**	**mean**
CD11c	80.70	266.29	94.14	275.21	95.22	319.34
MHCII	63.01	135.90	84.00	309.11	87.68	422.37
CD11c & MHCII	51.48	n/a	62.50	n/a	71.25	n/a
CD54 (ICAM-1)	73.48	443.50	77.33	667.53	86.74	874.74
CD86	95.88	844.04	96.19	979.47	96.78	1124.2
Gr1 (Ly6G)	25.84	n/a	26.96	n/a	24.74	n/a
CD45 (B220)	5.08	n/a	5.14	n/a	4.98	n/a

Table 4.1: FACS analysis of DC-enriched populations for surface antigens. Percentage of cells positively stained for each surface antigen is indicated. The mean level of fluorescence of positive populations is also indicated.

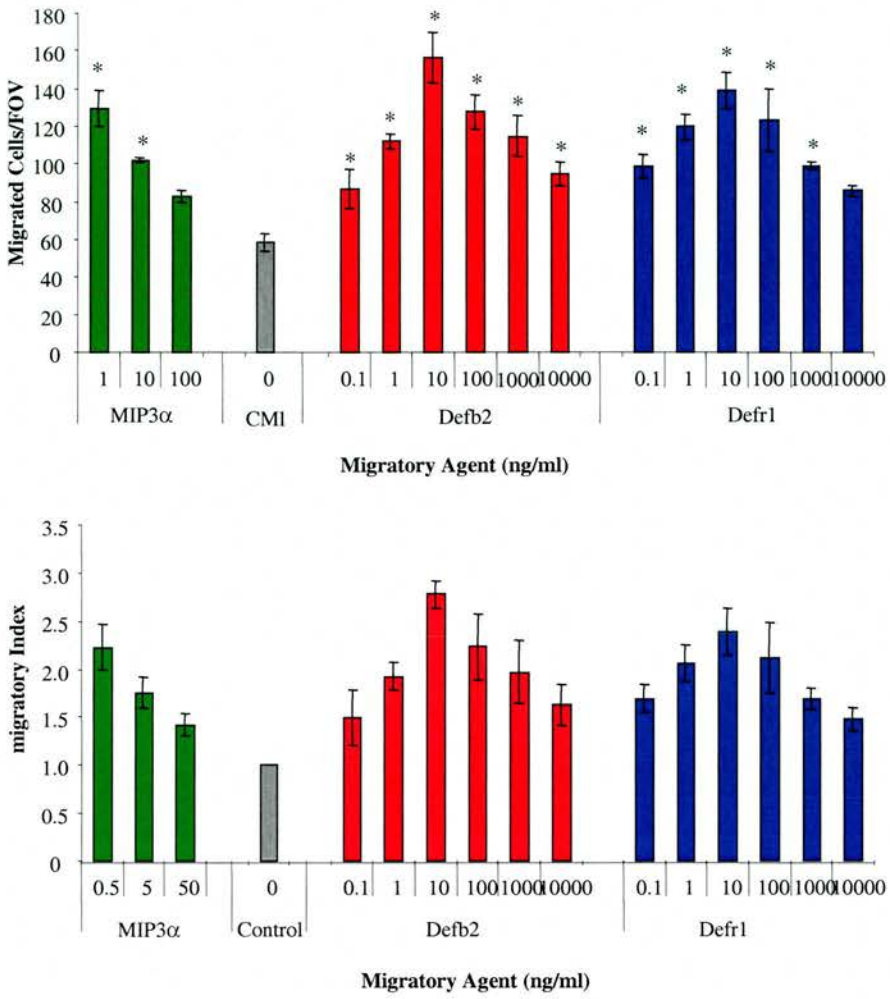

Figure 4.8: Migration of day 6 immature dendritic cells to the mouse β-defensins Defb2 and Defr1.

Dendritic cells were derived form bone marrow by culture with GM-CSF for 6 days. Cells were incubated in a 48-well chemotaxis chamber for 1.5 hours separated from different concentrations of Defb2 or Defr1 by a 5μm polycarbonate filter. MIP3α was used a positive control. The filter was stained and three fields of view were counted in each of three replicate wells. Graph A shows mean number of cells migrated per field of view form a total of nine random fields. Graph B shows the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the control (media alone) sample. * represents $p < 0.05$. Error bars represent the standard deviation of all nine replicate counts. Experiments were checked for reproducibility in a minimum of three experiments (*i.e.* n=3) , and a typical result is illustrated above.

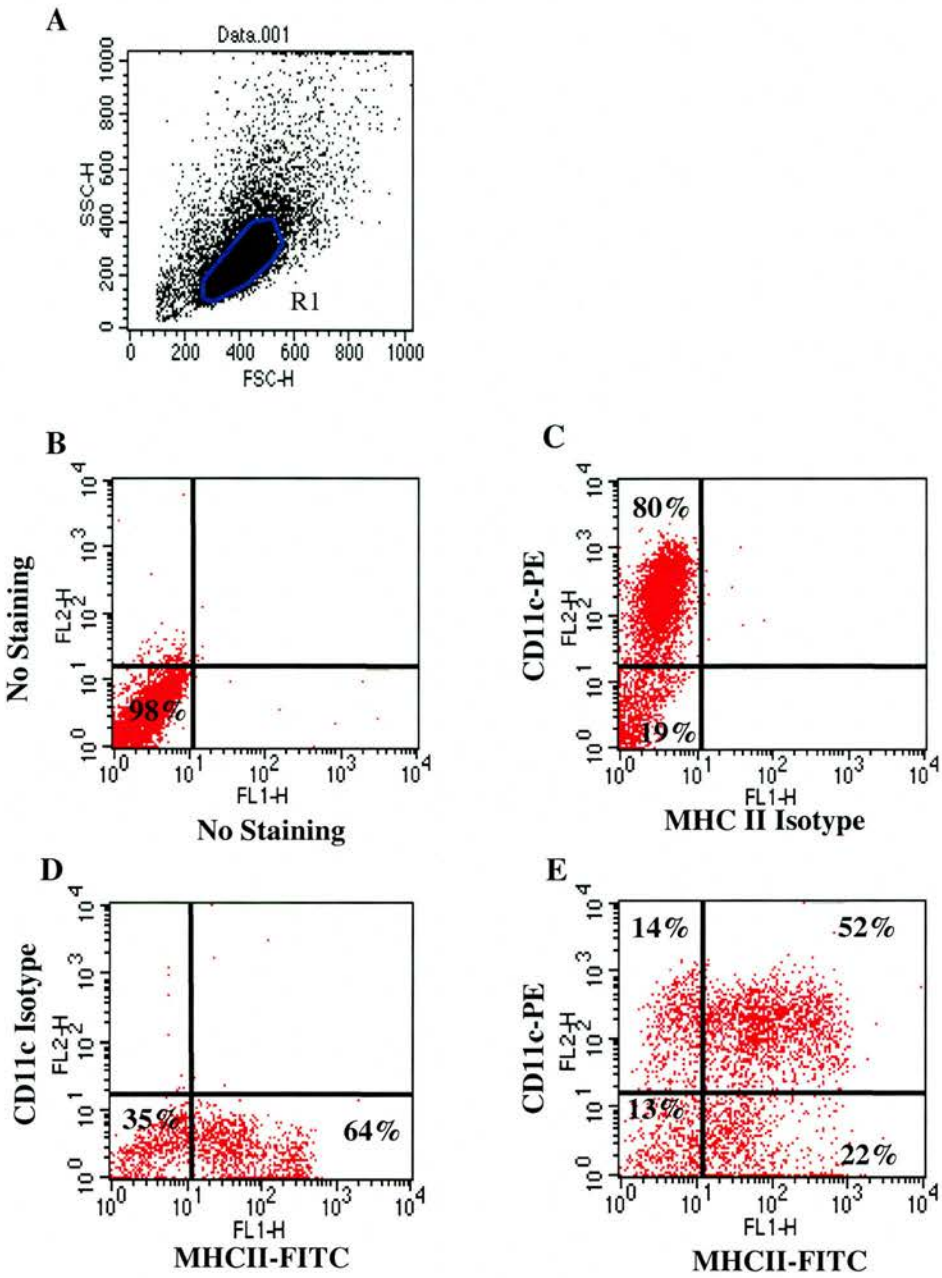

Figure 4.9: FACS analysis of day-6 immature dendritic cell surface antigen expression.

Bone marrow cells were isolated and cultured as previously described. The cells were double-stained for CD11c and MHCII. A) forward and side scatter and R1 gate used in subsequent analysis. B) Dot plot analysis showing unstained cells. C) Dot plot shows cells stained for CD11c. D) Dot plot analysis showing cells stained for MHC II expression. E) Dot plot analysis of cells double-stained for CD11c and MHC II. Percentages indicate the percentage of cells in each quadrant. All antibodies were either PE- or FITC-conjugated as indicated. Quadrant grid was set using isotype controls. FACs analysis was performed on all three replicate experiments to ensure reproducibility of the isolated cells phenotype (n=3).

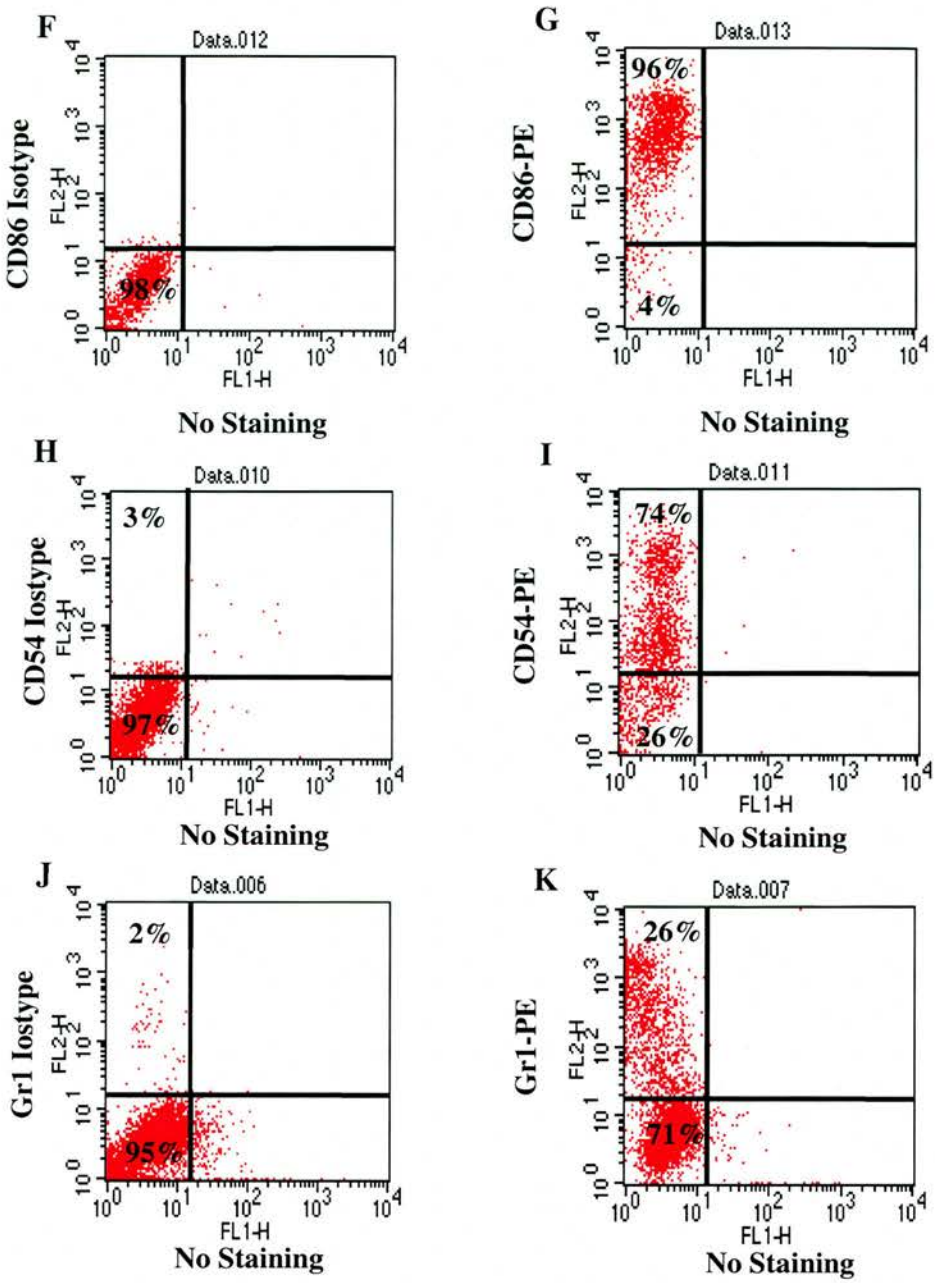

Figure 4.10: FACS analysis of day-6 immature dendritic cell surface antigen expression.

Bone marrow cells were isolated and cultured as previously described. The cells were double-stained for CD11c and MHCII. F) Dot plot analysis of cells stained with the isotype antibody for CD86. G) Dot plot showing cells stained for CD86. H) Dot plot analysis of cells stained with the isotype of CD54. I) Dot plot analysis of cells stained for CD86. J) Dot plot analysis of cells stained with the isotype for Gr1. K) Dot plot analysis of cells stained with the Gr1antibody. Cells were also stained for B220 (not shown). Percentages indicate percentage of cells in each quadrant. All antibodies were either PE- or FITC-conjugated as indicated. Quadrant grid was set using isotype controls. FACs analysis was performed on all three replicate experiments to ensure reproducibility of the isolated cells phenotype (n=3).

4.2.3 Chemoattractant Activity of Defb2 and Defr1 to Day-6 immature dendritic cells.

Total cells were isolated from the bone marrow of C57Bl/6 mice and cultured for 6 days in the presence of granulocyte/macrophage colony stimulating factor (GM-CSF) to generate a dendritic cell-enriched population. These cells were incubated in the presence of the peptides for 1.5 hrs and then stained with haematoxylin. Day 6 immature dendritic cells (d6iDC) showed significant migration in response to the positive control macrophage inflammatory protein-3alpha (MIP3α) at 1 ng/ml with an almost 4-fold increase in migration, an average of 75 cells/FOV were observed compared to ≈20 in the control wells (Figure 4.8).

Statistically significant ($p < 0.05$) migration by d6iDC was observed in response to Defb2 and Defr1 (Figure 4.8). The peak activity for Defb2 was observed to be between 10 and 100 ng/ml with approximately 160 cells/FOV compared to just under 60 in the control wells giving a MI of 2.75 at 10 ng/ml. At 100 or 1000 ng/ml the MI was 2.0, slightly reduced from the peak. The lowest migration was observed in response to 0.1 ng/ml with 85 an average of cells/FOV and a MI of 1.5. The peak migration in response to Defr1 was also observed to be between 10 and 100 ng/ml with approximately 140 cells/FOV at 10/ng/ml the migration at 100 ng/ml was more variable with ≈120 cells/FOV; these results give a peak MI of 2.2 to 2.5. The lowest level of migration was observed at 10000 ng/ml with a MI of ≈1.5 and 85 cells/FOV compared to the control.

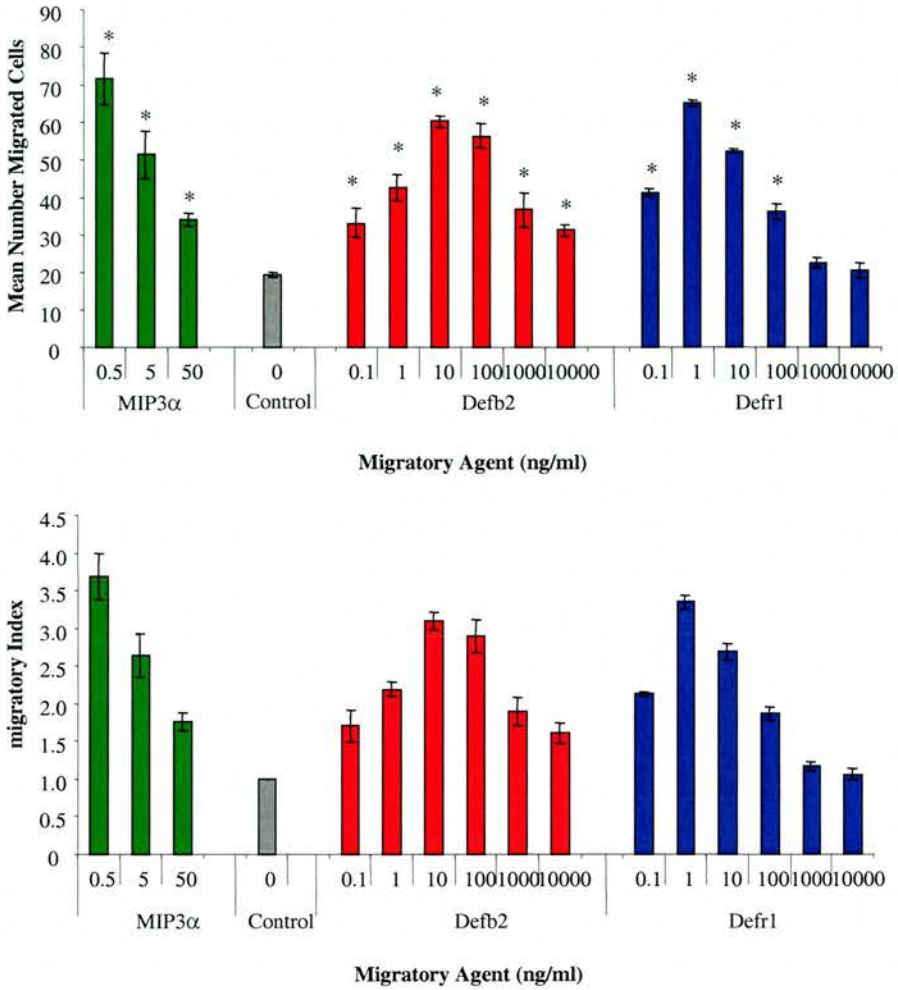

Figure 4.11: Migration of day 7 immature dendritic cells in response to the mouse β-defensins Defb2 and Defr1.

Dendritic cells were derived form bone marrow by culture with GM-CSF for 7 days. Cells were incubated in a 48-well chemotaxis chamber for 4.5 hours separated from different concentrations of Defb2 or Defr1 by a 5μm polycarbonate filter. MIP3α was used a positive control. The filter was stained and three fields of view were counted in each of three replicate wells. Graph A shows mean number of cells migrated per field of view form a total of nine random fields. Graph B shows the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the control (media alone) sample. * represents $p< 0.05$. Error bars represent the standard deviation of all nine replicate counts. Experiments were checked for reproducibility in a minimum of three experiments (*i.e.* n=3) , and a typical result is illustrated above.

FACS analysis of the cell population for various dendritic cell surface markers revealed that 51.5% of the cells were double positive for CD11c and MHMII, 73.5% expressed CD54 and almost 96% expressed CD86 (Figure 4.9 and 4.10, Table4.1). Contamination from granulocytes was 25% and B-cell contamination, assessed by B220 expression, was approximately 5%. As these cell types are the main contaminant, these results suggest that approximately 70% of the population were dendritic cells. The observed compositionof the DC-enriched population is in agreement with previous findings using this culture method (Personal communication, Dr Sarah Howie, University of Edinburgh).

4.2.4 Chemoattractant Activity of Defb2 and Defr1 to day-7 immature dendritic cells.

The migratory response of dendritic cells that had been cultured in the presence of GM-CSF (d7iDC) was also assessed to investigate if culture-period or developmental stage of the dendritic cells affected the migration in response to Defb2 or Defr1. Migration in response to macrophage inflammatory protein-3 alpha (MIP3α) at 1 ng/ml was increased compared to the day 6 DCs, with a MI of 4.5, largely as a result of a lower background migration (Figure 4.5). The peak level of migration of d7iDCs in response to Defb2 was again between 10 and 100 ng/ml, the MI of 3.0 is marginally higher than was observed with d6iDCs. The lowest level of migration was seen at 0.1 and 10000 ng/ml, where an average of just over 30 cells/FOV were observed compared to 20 in the control wells. The concentration of Defr1 that induced the highest level of migration was 1 ng/ml. This is lower than the 10 ng/ml that gave the peak concentration of migration for d6iDC. The

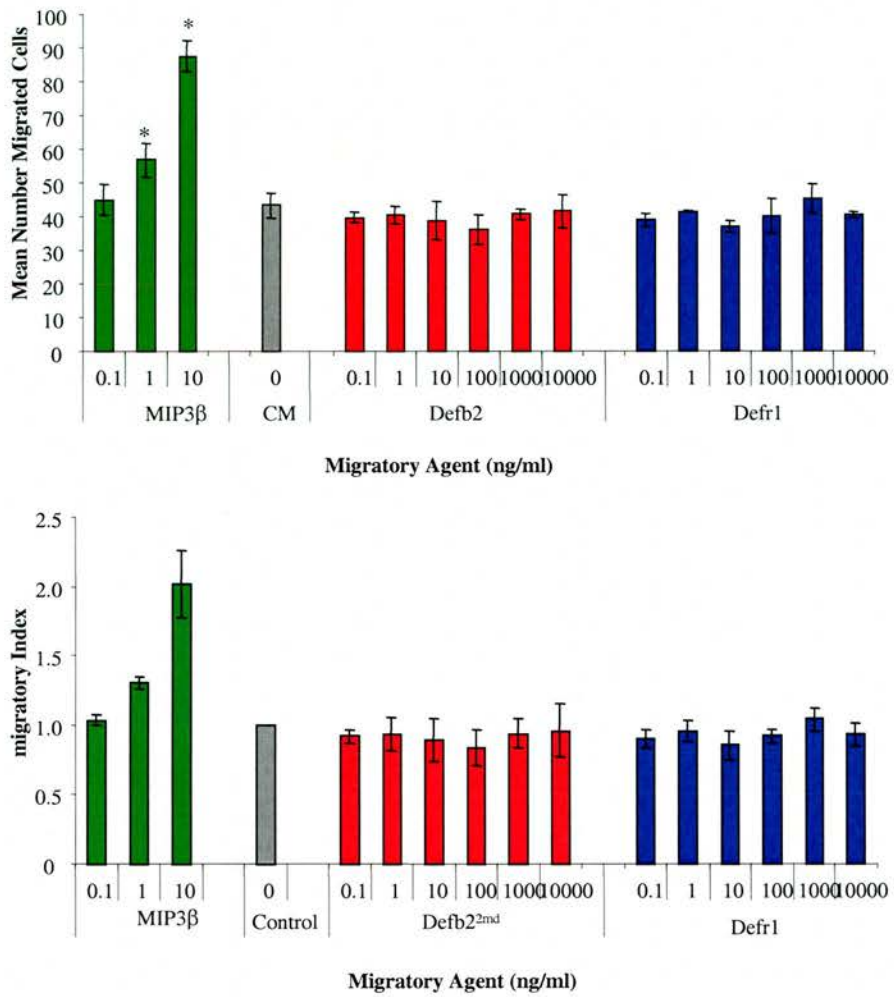

Figure 4.12: Migration of mature dendritic cells in response to the mouse β-defensins Defb2 and Defr1.

Dendritic cells were derived form bone marrow by culture with GM-CSF for 7 days and matured with LPS. Cells were incubated in a 48-well chemotaxis chamber for 4.5 hours separated from different concentrations of Defb2 or Defr1 by a 5μm polycarbonate filter. MIP3β was used a positive control. The filter was stained and three fields of view were counted in each of three replicate wells. Graph A shows mean number of cells migrated per field of view form a total of nine random fields. Graph B shows the migratory index, the number of cells migrated in each sample divided by the number of migrated cells in the control (media alone) sample. * represents $p< 0.05$. Error bars represent the standard deviation of all nine replicate counts. Experiments were checked for reproducibility in a minimum of three experiments (*i.e.* n=3) , and a typical result is illustrated above.

level of migration was also slightly higher than that observed for Defb2, with a MI of almost 3.5. Defr1 at a concentration of 0.1 ng/ml also induced significant migration with a MI of 2.5. Defr1 at a concentration of 1000 and 1000 ng/ml did not induce statistically significant migration; all other concentrations of Defr1 and all concentrations of Defb2 tested did induce statistically significant migration compared to the control ($p < 0.05$). Moreover, there were statistically significant differences in the levels of migration induced by Defb2 and Defr1 were observed at concentrations of 1 ng/ml and 100 ng/ml ($p < 0.05$).

FACS analysis of the cell population revealed that the percentage of cells positive for MHMII and CD11c was 69.5; this is significantly higher than was seen with d6iDC (Table 4.1). Moreover, the percentage of cells that were positive for CD11c or MHMII and the mean fluorescence were also higher than for d6iDC, suggesting that level of expression of these surface markers were also higher. The percentage of cells positive for CD86 or CD54 were approximately the same as for d6iDC, but the mean fluorescence was in each case higher, suggesting that the level of expression of the surface antigens was higher. The level of Gr1+ Granulocyte and B-cell contamination were similar to those seen in the d6iDC populations.

4.2.5 Chemoattractant Activity of Defb2 and Defr1 to mature dendritic cells.

Bone marrow cells were isolated and cultured as above in section 4.2.4, except that 24 hrs prior to the experiment LPS was added to the culture media to induce maturity. Mature dendritic cells (mDC) did not show

significant migration in response to either Defb2or Defr1 (Figure 4.52). However, in response to the positive control a statistically significant, but low level, of migration was observed with ≈85 cells/FOV compared to just over 40 in the control, giving a MI of 2.0.

FACS analysis was conducted as before (Table 4.1). This revealed a population that has a higher mean fluorescence in the populations of cells expressing CD11c or MHC II. A greater percentage of cells that were double positive for MHC II and CD11c. CD86 and CD54 levels were also elevated compared to immature DCs, whereas levels of contamination by granulocytes (Gr1+) or B-cells (B220) were similar to those observed in previous populations.

4.3 Discussion

4.3.1 Introduction

Previous studies have revealed that many cationic peptides, such as the cathelicidin LL-37 and human α- and β-defensins, possess chemoattractant properties and can induce migration in a variety of cell types reviewed in (Yang *et al.*, 2000a; Yang *et al.*, 2001). The first report of a chemotactic activity of defensins was from an investigation of the monocyte-chemotactic activity of neutrophils (Territo *et al.*, 1989). Territo *et al.* showed that the chemotactic activity of fractionated extracts from neutrophil granules was largely contained within the α-defensin fraction, and subsequent analyses with the purified neutrophil α-defensins indicated these to be chemotaxins for monocytes and to a lesser extent for neutrophils. However, these migratory responses were not repeated in an *in vitro* study by Chertov *et al.* (Chertov *et al.*, 1996), although this study does record *in vivo* migration of neutrophils. The Chertov *et al.* study was performed with a 48-well chemotaxis chamber, whereas the study conducted by Territo *et al.* (1989) is based on assays of the leading front cells migrating into a filter. It is possible therefore that the discrepancy is due to technical differences between the two assays. Subsequent studies have supported and further characterised the range of chemoattractant activities of defensins. The human α-defensins, human neutrophil peptides 1-2 (HNP1-2) have been shown to chemoattract naïve, but not memory, CD4+ T-cells and CD8+ T-cells (Yang *et al.*, 2000a). In contrast the human β-defensins DEFB1 and 2 attract memory, but not naïve, CD4+ T-cells (Yang *et al.*, 1999). In addition human α- and β-defensins have also been shown to attract monocytes and immature but not mature dendritic

cells (Yang *et al.*, 1999; Yang *et al.*, 2000a; Garcia *et al.*, 2001a). These previous studies have been performed with human defensins and to date a chemoattractant property has not been reported for murine β-defensins. Although one study showed that Defb2 and Defb3 fused to lymphoma antigen, sFv, were able to induce migration of immature but not mature dendritic cells (Biragyn *et al.*, 2001).

4.3.2 Migration of Neutrophils

This chapter presents data on the ability of synthetic murine β-defensins Defb2 and Defr1 to induce cellular migration in neutrophils, CD4+ T-cells and dendritic cells *in vitro* using a 48-well microchemotaxis chamber (Summarised in Table 4.2). The studies carried out on CD4+ T-cells and dendritic cells were performed in collaboration with Dr Sarah Howie and Professor Johnathan Lamb at the University of Edinburgh. The results show that *in vitro* Defb2 and Defr1 possess the ability to induce migration in CD4+ T-cells, and immature bone marrow derived-dendritic cells but that this activity is lost upon maturation. Neutrophils were not found to migrate in response to Defb2 or Defr1 (Table 4.2).

	Defb2			Defr1		
Cell Type	**Migrn.**	**Peak MI**	**Conc.**	**Migrn.**	**Peak MI**	**Conc.**
Neutrophils	×	-	-	×	-	-
$CD4^+$ T-cells	√	5.0	10	√	4.5	1
d6iDC	√	2.75	10-100	√	2.2-2.5	10-100
d7iDC	√	3.0	10-100	√	3.5	1
mDC	×	-	-	×	-	-

Table 4.2: Summary of cell migration induced by murine β-defensins. Migrn. indicates whether of not the β-defensin induced statistically significant migration of each cell type ($p < 0.05$). Peak indicates the maximum migration index (MI) of each cell type, and Conc. shows the concentration of β-defensin (in ng/ml) that induced the maximum migration.

Neutrophils contribute to innate host defence in response to inflammatory signals by migrating along chemoattractant gradients to sites of inflammation where they phagocytose opsonised particles and degranulate releasing granule contents, which includes the α-defensins, HNP1-4. In the current study, the analysis of neutrophil migration in response to β-defensins indicates that neither Defb2 nor Defr1 are chemoattractants for neutrophils between concentrations of 0.1 and 10000 ng/ml. This observation is in broad agreement with previous observations that β-defensins do not induce migration of neutrophils; separate studies of DEFB103 and DEFB104 found that neither possessed chemotactic activity for neutrophils (Garcia *et al.*, 2001a; Garcia *et al.*, 2001b). As discussed above, α-Defensins have been shown to induce a low level of migration in neutrophils, in an *in vitro* assay purified HNP-2 and -3 caused an approximately 20% increase in migration at a concentration 0.1 μM (Territo *et al.*, 1989). Furthermore, in a separate study Chertov *et al.*, showed that a subcutaneous injection of 1 μg of HNP-1 into BALB/c mice resulted in an accumulation of monocytes and neutrophils by 4 hrs (Chertov *et al.*, 1996). It is possible that whilst neither α- nor β-defensins are directly chemotactic for neutrophils and thus display little or no such activity *in vitro,* whereas *in vivo* they may induce a cascade of proinflammatory signals resulting in the accumulation of neutrophils. Chemokines that do attract neutrophils include interleukin 8 (IL-8); this specifically attracts neutrophils and has no migratory effect on either lymphocytes or monocytes. It is interesting to note that IL-8 has been shown to cause release of the α-defensins from neutrophil granules (Chertov *et al.*, 1996) and neutrophil defensins have been shown stimulate IL-8 synthesis in airway epithelial cells (van Wetering *et al.*, 1997b). It may be possible therefore that the neutrophil chemoattractant activity of HNPs is mediated

via IL-8, this would explain the observation of chemotaxis *in vivo* and the failure of Chertov *et al.* to observe migration *in vitro.* For example, in another study injection of between 4 ng and 4 μg of HNP-1 into the peritoneum of experimentally *K. pneumoniae*-infected in mice resulted in a significant reduction in bacterial survival compared to saline treated controls. This activity did not appear to be due to the direct antibacterial effect of HNP-1, but due, at least in part, to the chemotactic response of lymphocytes, macrophages and granulocytes such as neutrophils (Welling *et al.*, 1998). Therefore, a subcutaneous injection in mice of Defb2 or Defr1 may lead to an accumulation of neutrophils via secondary signalling roles, even though they do not possess direct chemoattractant ability for neutrophils *in vitro.*

4.3.3 Migration of $CD4^+$ T-lymphocytes

CD4+ T-lymphocytes were isolated from mouse spleens and purified using the autoMACS system (Miltenyi Biotec), this gave raise to a population of cells at least 90% positive for CD3 and CD4. In the current study, CD3+/CD4+ T-lymphocytes showed significant levels of migration in response to both Defb2 and Defr1 (Table 4.2). The peak migration in response to Defb2 was 10 ng/ml, whereas Defr1 induced maximal migration at 1 ng/ml. There was, however, no significant difference between the maximal level of migration induced by Defb2 and Defr1, both induced a 4.5 to 5-fold increase in migration compared to media alone and this was slightly higher than the migration induced by the positive control stromal cell-derived factor 1 alpha (SDF1α). This chemokine, which has also been shown to induce chemotaxis of monocytes and B- and T-lymphocytes, but not neutrophils, signals through the chemokine receptor CXCR4. The studies presented here do not investigate which receptor is mediating the CD4+ cell

migration, and this would be an interesting area of future study. The data presented in this chapter are in broad agreement with previous data showing that both human α- and β-defensins can act as chemoattractants for CD4+ T-cells. However, in previous studies a dichotomy was observed in the subsets of T-cells that respond to α and β-defensins. The α-defensins HNP-1 and -2 induced migration in CD4+/CD45RA+ naïve T-cells (Yang *et al.*, 2000a). In contrast, the β-defensins induced attracted CD4+/CD45RO+ memory T-cells (Yang *et al.*, 1999). The level of migration of CD4+ memory T-cells in the Tang et al. study in response to DEFB4 was maximal at 1000 ng/ml, with a 5-fold increase cell migration compared to media alone. This response is greater than that seen in this study, however, Tang *et al*, analyses were conducted with memory T-cells as opposed to the more mixed population of CD4+ T-cells used in this study, which would have contained memory and naïve CD4+ T-cells. Therefore, the potential response of the memory T-cells may have been diluted by the failure of the naïve T-cells in the population to respond.

The data presented in the current study do not distinguish between the responses of the distinct T-cell subsets and consequently it is unclear whether Defb2 and Defr1 are targeting one subset or both naïve and memory T-cells and further studies are required to investigate this issue. It may however be possible to identify the subset of $CD4^+$ T-cells that have migrated *in situ* (*i.e.* on the filter itself) by staining with antibodies specific to cell surface markers that identify the different subsets. If it is necessary to perform chemotaxis experiments using separate populations of naïve and memory T-cells these can be isolated in several ways, although such studies may be hindered by difficulties in isolating the sufficient numbers of cells. It may be possible to separate naïve and memory T-cells based on CD62L expression. CD62L (L-

selectin) is highly expressed on naïve murine T-cells, but expressed at much lower levels on memory T-cells. CD62L is also expressed by B-lymphocytes, neutrophils, eosinophils and monocytes; therefore, selection for high or low expression of CD62L would have to be preceded by selection for CD4+ cells and this may significantly increase the number of animals required. Another method for isolation of the distinct CD4+ T-lymphocytes populations would be to use expression of CD45RB; this surface marker is expressed at high levels on by naïve T-cells ($CD45RB^{hi}$) compared to memory T-cells, which have the phenotype $CD45RB^{lo}$ (Seder and Paul, 1994). Although selection for CD4 expression would have to be conducted prior to selection for naïve and memory cells and thus a large number of animals may be required. Separation of CD4+ T-cells based on CD45/RB expression could be conducted using fluorescence-activated cell sorting (FACS).

The different responses of memory and naïve T-cells to β-defensins is likely to be due to expression of distinct chemokine receptors on the different population subsets. Indeed as was discussed above, β-defensins have been show to signal through CCR6 in both humans and mice (Yang *et al.*, 1999; Biragyn *et al.*, 2001), whereas the HNP-1 and -2 do not (Yang *et al.*, 2000a). Chemokines are small highly conserved molecules, which typically contain four cysteines and like defensins, they are classified into two subfamilies depending arrangement of those cysteines. α-Chemokines such as interleukin-8 (IL-8) and SDF1α have a C-X-C motif, where there is an extra amino acid separating the first and second cysteines. α-Chemokines signal by interaction with C-X-C chemokine receptors (CXCRs); whereas the β-chemokines, however, such as MIP3α and MIP3β contain a C-C motif, and there is no amino acid separating the first and second cysteines, these chemokines signal through C-C chemokine receptors (CCRs) reviewed in

(Murphy, 1994). It is interesting to note that the α-defensin consensus sequence shows the presence of a CXC motif and CC motif, whereas the β-defensins consensus sequence contains only a CC motif, Defb2 and Defr1 also have a CC motif, but no CXC motif (Figure 6.8). Moreover, β-chemokines, like the β-defensins, do not induce migration in neutrophils, whereas α-chemokines do. Therefore, it is tempting to speculate that the different migratory responses of T-cells to α- and β-defensins may not only be due to different receptors - with the α-defensins signalling via a CXC-receptor and β-defensins acting by a CC-receptor, such as CCR6 (Yang *et al.*, 1999; Biragyn *et al.*, 2001). The receptor through which α-defensins signal is yet to be elucidated, and moreover as α-defensins possess both a CC and CXC motif they may signal through either CC-receptors or CXCRs. It may be possible to explore the issue of receptor use by antibody blocking experiments; $CD4^+$ cells or dendritic cells could be incubated with antibodies designed to block specific chemokine receptors and then exposed to defensins to see if cell migration still occurs in the absence of a 'free' and functional receptor.

The biological relevance of the observation that the two different subfamilies of defensins attract different subsets of T-cells also remains unclear. It has been argued that HNPs are involved in systemic defence, whereas the human β-defensins DEFB1 and DEFB4 play a role in protecting specific epithelial tissues (Zhao *et al.*, 1996). It has also been predicted that memory T-cells recirculate preferentially in the tissues where their immunological memory was first generated, whereas naïve T-cells will circulate more widely throughout the body (Moser and Loetscher, 2001). This may provide a possible explanation for the different T-cell subsets attracted by α- and β-defensins. The β-defensins, mainly expressed at epithelial tissues may play a

α-Chemokines:

IL-8 SAKELRCQCIKTYSKPFHPKIKELRVIESGPHCANTEIIVKLSDGRELCLDPKENWVQRV VEKFLKRAENS

α-Defensins:

HNP-1 VVCACRRALCLPRERRAGFCRIRGRIHPLCCRR

β-Chemokines:

hMIP3α ASNFDCCLGYTDRILHPKFIVGFTRQLANEGCDINAIIFHTKKKLSVCANPKQTWVKYIV RLLSKKVKMN

β-Defensins:

DEFB4 DPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP

DEFB1 DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK

Defb2 ELDHCHTNGGYCVRAICPPSARRPGSCFPEKNPCCKYMK

Defr1 DPVTYIRNGGICQYRCIGLRHKIGTCGSPFKCCK

Figure 4.13: α-Chemokines and α-defensins possess a CXC motif. β-chemokines, β-defensins and α-defensins possess a CC motif. Cysteines are highlighted in bold and CXC and CC motifs are underlined. Residues said to be conserved between MIP3α and the β-defensins are highlighted in red, blue and green. Green and red show the conservation of aspartate and leucine residues (or amino acids with similar properties) between MIP3α and DEFB1 and 4. Residues in blue are the amphipathic amino acids that are proposed to be conserved between MIP3α and DEFB1 and 4 (corresponding amino acids are shown for Defb2 and Defr1).

role, in conjunction with other more tissue-specific chemokines, in maintaining a physiological 'through-traffic' of antigen-relevant memory T-cells for immediate response The α-defensins, which may play a more systemic role, may act to attract naïve T-cells to the site of inflammation. Therefore, it would be of interest to investigate whether a particular β-defensin shows an increased capacity to attract memory T-cells that were generated in a tissue where it is expressed compared to a tissue where it is not. For example, does Defr1 has more potent chemoattractant activity for memory T-cells generated in the testis, where it is expressed at high levels, compared to those generated in the lungs, where Defr1 expression is not detected? As Defr1 and Defb2, which have very different patterns

of expression, induced similar levels of migration, it is perhaps unlikely that β-defensins play an important or direct role in migration of tissue-specific memory T-cells; and they may instead play a role in maintaining levels of more migration of more generalised memory T-cells. However, differences in the induction of these two genes, may serve to regulate cell migration in a more specific manner.

4.3.4 Migration of Dendritic Cells

Dendritic cells are professional antigen presenting cells that are found in all lymphoid and non-lymphoid organs. In an immature state, they constantly sample the environment by endocytosis and phagocytosis, but antigenic peptide-MHC II complexes accumulate in the lysosomes. However, upon exposure to bacterial products such as lipopolysaccharide (LPS) or cytokines such as tumour necrosis factor alpha (TNF-α), endocytosis is down regulated and peptide-MHC II expression on the cell surface is up-regulated reviewed in (Watts, 1997). This means that an immature dendritic cell samples from the environment but does not display antigen, but on exposure to bacterial antigens such as LPS results in a massive up-regulation in the expression of antigen-MHC II complexes on the cell surface.

The work presented in this chapter also analyses the migration of dendritic cell-enriched populations in response to Defb2 and Defr1 (Table 4.2). Dendritic cells (DCs) were isolated from mouse bone marrow cells and cultured in the presence of 500 - 1000 U/ml of granulocyte/macrophage colony-stimulating factor (GM-CSF) following a protocol modified from the procedure established by Inaba *et al* (Inaba *et al.*, 1992). This protocol generated a DC-enriched population, which FACS analysis demonstrated to

consist of approximately 70% dendritic cells and the main contaminants were Gr1+ granulocytes. This population composition is in broad agreement with previous studies using this technique.

The DC-enriched populations described above were used to examine the ability of Defb2 and Defr1 to induce migration of dendritic cells. Previous data on the migration of DCs in response to human α- and β-defensins showed that dendritic cells lose their responsiveness to defensins upon maturation (Yang *et al.*, 1999; Yang *et al.*, 2000a). Initially therefore the migratory activity of DCs in response to Defb2 and Defr1 was analysed after 6 days of culture with GM-CSF and termed day-6 immature dendritic cells (d6iDC). Both β-defensin peptides were observed to induce migration in d6iDCs, the peak migration was observed between 10 and 100 ng/ml and resulted in a ≈2.25 to ≈2.75-fold increase in migration in response to Defb2 and an approximately 2 to 2.5-fold increase in migration in response to Defr1. This level of migration was less than that which was observed for CD4+ T-cells, but is similar to the level of migration seen in the previous defensin-induced migration of dendritic cells (Yang *et al.*, 1999; Yang *et al.*, 2000a). Subsequent studies also analysed the migratory responses of immature dendritic cells cultured for 7 days (d7iDC). The migration induced by d7iDCs was very similar to that observed with d6iDC, in response to Defb2 the maximal migratory activity, which was induced by 10 to 100 ng/ml. However, maximal chemotaxis in response to Defr1 was induced by 1 to 10 μg/ml and this is lower than the Defr1 concentration that induced maximum migration of d6iDC, suggesting that the sensitivity of DCs for Defr1 may alter with stage of development or age. The peak level of migration by either d6iDCs or d7iDC in these studies is lower than that observed by Tang *et al.* (Yang *et al.*, 1999) who observed a 5-fold induction in chemotaxis in response

to DEFB4, with background migration levels similar to those seen with d7iDCs in these studies. However, 10 ng/ml of DEFB4 in the Tang *et al.* study induced a 3-fold induction in migration. This is similar to the level of migration induced by Defb2 in this study. These differences may very well be due to differences between the activities of the human and murine β-defensins, or alternatively could be the result of differences in the DCs used; for example, Tang *et al.* used CD34+ progenitor-derived cells, whereas the cells from the current study were derived from bone marrow cells. The levels of migration seen in this current study are, however, similar to the migration of DCs observed in response to α-defensins (Yang *et al.*, 2000a). In the study by Biragyn *et al.* they observed a 6-fold induction in migration in response to 10 ng/ml of Defb2 fused to the sFv lymphoma antigen, but an adjuvant effect and increased migratory potency in the fusion protein compared to the native form can not be ruled out (Biragyn *et al.*, 2001).

As discussed above, the fold-induction of migration in response to Defb2 and Defr1 were similar for d6iDC and d7iDC. However, the actual number of cells that migrated (in terms of mean number of cells per field of view) was lower for d7iDC than for d6iDC. Therefore, the low background migration and migratory indices of the d6iDC and d7iDC obscure the fact that d7iDCs appear to be less migratory in terms of actual numbers of cells migrating. This may suggest that while d7iDC have a lower migratory potential than d6iDC, they do however retain the ability respond to defensins by inducing migration. It must also be acknowledged that the variation between the replicate studies for both the d6iDCs and d7iDCs was quite high, which not only made statistical comparison between replicate experiments difficult. Further replicate experiments, or a more reproducible and rigorous source of DCs may be required (see below).

The migratory response of mature dendritic cells (mDCs) was also analysed as part of this study. Exposure to antigens has been shown to induce maturation in dendritic cells, and for the purposes of this study, d7iDCs were matured by culture with 100 ng/ml lipopolysaccharide (LPS) for the 24 hrs prior to use. The current study found that mDCs did not migrate in response to either Defr1 or Defb2. This is in agreement with previous studies that demonstrated that upon maturation with TNFα DCs lose their responsiveness to α- and β-defensins (Yang *et al.*, 1999; Yang *et al.*, 2000a). mDCs also lost the ability to migrate to Defb2-sFv fusion molecule in the Biragyn *et al.* study (Biragyn *et al.*, 2001). It has been suggested that β-defensin-induced migration of both human and murine DCs is mediated via CCR6 (Yang *et al.*, 1999; Biragyn *et al.*, 2001). Furthermore, it has been shown that following maturation, DCs down-regulate expression of CCR6 and up-regulate expression of another chemokine receptor CCR7, correspondingly they cease to migrate in response to MIP3α, which signals via CCR6, and show migratory responses to MIP3β, which signals through CCR7 (Dieu *et al.*, 1998). Therefore, mDCs, which no longer express CCR6, are also no longer chemoattracted by β-defensins. This receptor-switch permits mDCs, which having matured, have massively up-regulated antigen presentation on their surface to leave the inflamed or infected tissue and, in response to a different set of chemokines, migrate to the lymph nodes to prime naive T-cells reviewed in (Banchereau and Steinman, 1998).

The purity of the dendritic cell populations was assessed by FACS using a panel of antibodies. The cell populations used in this study were analysed for expression of CD11c and MHC-II, which together are markers of dendritic cells, and the CD86 co-stimulatory molecule and CD54/ICAM-1,

which are also markers of DCs; the populations were also analysed for contaminating granulocytes and B-cells. Generally, all populations used were found to be in broad agreement with previous studies using this method (Dr Sarah Howie, University of Edinburgh). The DC populations used in these studies were shown by FACS analysis to contain approximately 25% of cells positive for the granulocyte marker Gr1 (Ly6G); this relatively high level of contamination demonstrates that these populations are 'dendritic cell-enriched populations' rather than a pure dendritic cell population. Contamination by B-cells was found to be present but at much lower levels of ≈5%. The majority of the granulocytes are likely to be neutrophils, which as described above do not migrate in response to Defb2 or Defr1 so they would not contribute to the chemotaxis. However, the contribution of B-cells, and other granulocytes such as eosinophils to the observed migration is not known, although a previous study have indicated that eosinophils do migrate in response to DEFB104 (Garcia *et al.*, 2001b). DCs produced by the Inaba *et al.* method upon which this technique is based expressed CD11c and MHC class II (Inaba *et al.*, 1992). All DC populations used in this study expressed both CD11c and MHC-II, moreover, the expression of both of these molecules increased with the age and maturity of the DC populations and this finding is also in agreement with previous findings, as mDCs up-regulate expression of MHC-II on their surface (Inaba *et al.*, 1992; Yang *et al.*, 2000a). The DCs used in this study did express very high levels of CD86 and CD54 which are normally only expressed at high levels following maturation. However, it is important to note that whilst the percentage of cells expressing these two markers did not increase with maturation, the mean level of fluorescence did increase which suggests that cells are expressing these markers at higher levels. The reason for the high levels of CD86 and CD54 expression remains unknown, the day-6 and day-7

DCs were still immature as they responded to MIP3α and mature DCs do not. One possible explanation may be strain differences between the mice used in the different studies. The Inaba *et al.* (1992) study used BALB/c, mice whereas the studies presented in this chapter used C57Bl/6 mice. It is possible that DCs from the two strains develop at different rates are therefore upon analysis different levels of surface markers are detected.

As was discussed above, the replicate experiments conducted in this study with bone marrow derived dendritic cells gave quite variable results. This may have been due to natural variation in the migratory responses of DCs, but it may also be due to the *in vitro* culture method employed to generate a population of DCs. As the DCs are developing from progenitors, they may develop at different rates from culture to culture and this may lead to differences in the migration observed in response to the β-defensins. As was described in the Material and Methods section, bone marrow cells were isolated from the tibias and femurs of mice and cultured in the presence of GM-CSF based on the technique established by Inaba *et al.* (Inaba *et al.*, 1992). Culturing bone marrow progenitor cells with GM-CSF stimulates the development of not only dendritic cells, but also granulocytes (hence the relatively high levels of contamination) and macrophages (Inaba *et al.*, 1992). Cultures were established on day 0, and fed on days 3 and 6, with gentle swirling of the plate to remove non-adherent granulocytes. The granulocytes are non-adherent cells, the macrophages strongly adhere to the culture dish, whereas the dendritic cells adhere more weakly, this permits the DCs to be removed by gentle pipetting.

In the original Inaba *et al.* method after 4 days of culture, the aggregates of proliferating less-mature dendritic cells selected away from the stroma and

returned to culture. Moreover, the Inaba *et al.* (1992) study and a separate study by Biragyn *et al* (Biragyn *et al.*, 2001) depleted contaminating cells prior to culture of the bone marrows cells with GM-CSF. These steps were omitted in the current studies, and their re-introduction may increase the purity of the population. This can be done by incubating freshly isolated bone marrow cells with cocktails of antibodies against B-cells and MHC II antigens plus complement (Inaba *et al.*, 1992). Therefore, if the above studies are repeated using DCs that were selected away from the stroma the result may be a more pure population of dendritic cells and consequently more reproducible results. Other techniques for culturing of DCs from mouse bone marrow have included IL-4 with GM-CSF in the culture media (Yang *et al.*, 2000a; Biragyn *et al.*, 2001), this was not included in the studies described here and its incorporation in future experiments may improve the purity of the population.

4.3.5 β-Defensins as Chemokines

The observation that Defr1 also induced migration in of $CD4^+$ T-cells and DCs is interesting in light of the fact that Defr1 appears to be constitutively expressed. Although Defr1 expression may be induced by an, as yet, unknown stimulus as the upstream sequence of the gene does contain an NF-κB binding site (Morrison *et al.*, 2002a). However, the constitutively expressed DEFB1 has also been shown to induce migration of HEK-293 cells transfected with the chemokine receptor CCR6 (Yang *et al.*, 1999). Chemokines can also show constitutive or inducible patterns of expression. Constitutively expressed chemokines, also referred to as homeostatic chemokines, function within discrete microenvironments and are thought to

be specialized for maintaining a low level of immune cells trafficking through tissues, whereas the inducible or inflammatory chemokines function to recruit cells in an inflammatory response. It is possible therefore, that constitutive (DEFB1 and Defr1) and inducible (DEFB4 and Defb2) β-defensins may show similar patterns of activity. It is also interesting that Defr1 functions as a chemoattractant factor, when it has lost the first canonical cysteine present in other β-defensins (Morrison *et al.*, 2002a), and also given that the synthetic form used in these studies is known to exist in dimeric and monomeric forms. It would be of interest to investigate whether any differences exist between the chemotactic abilities of the Defr1 monomer and dimer, although the CC motif is present in both structural forms.

The structure and charge distribution of β-defensins has been proposed to be important for the ability of DEFB1 and DEFB4 to bind the CCR6 receptor (Bauer *et al.*, 2001; Perez-Canadillas *et al.*, 2001; Hoover *et al.* 2002). The NMR structures of the human and murine MIP-3α chemokine, which specifically binds to the CCR6 receptor, were recently elucidated (Perez-Canadillas *et al.*, 2001; Hoover *et al.* 2002). A comparison of the structure of MIP3α with that of DEFB4 highlighted the presence of a highly symmetric distribution of positive charges and the conservation of an aspartate and a leucine residue at the amino termini of the peptides (Figure 4.13, residues highlighted in green and red). Indeed the authors describe the DEFB4 three-dimensional structure as a 'simplified form of MIP3α' (Perez-Canadillas *et al.*, 2001). They suggest that as for the DCCL region of MIP3α, the aspartate-leucine region of DEFB4 may play a role ion receptor binding, activation and signalling (Perez-Canadillas *et al.*, 2001; Hoover et al., 2002). The aspartate residue is also conserved DEFB1, but the leucine is replaced by a valine. However, both leucine and valine are amphipathic amino acids and the valine may therefore

retain some of the properties of the leucine residue (Bauer *et al.*, 2001). Interestingly, some of these features may also be conserved in Defb2 and Defr1. For example, Defr1 retains the aspartate seen in DEFB1 and DEFB4, but has an isoleucine in the position occupied by leucine in MIP3α and DEFB4. In Defb2, the aspartate residue is replaced by the amino acid glutamate, which like aspartate is acidic. However, a histidine residue replaces the valine and leucine residues in DEFB1 and DEFB4 respectively.

A very recent study compared the structures of DEFB4 and human MIP3α (Hoover *et al.*, 2002). The authors found that in the three-dimensional structure of MIP3α the leucine residue is 0.85 nm form the aspartate residue. Hoover *et al.* also noted that within the same distance of the aspartate residues of DEFB1 and DEFB4 there are the amphipathic amino acids tyrosine (DEFB1) and valine (DEFB4) (Figure 4.13, residues highlighted in green and blue). They argue that the D-Y and D-V of DEFB1 and DEFB4 respectively are the functional equivalents of the DCCL region in MIP3α. It is interesting to note that in the corresponding positions in Defr1 there is a valine, whereas in Defb2 there is anther aspartate residue. Elucidation of the three-dimensional structures of Defr1 and Defb2 and subsequent mutational analysis may help to understand the role of these residues in the interaction of β-defensins with the chemokine receptor.

It is noteworthy that in comparison with the antibacterial activity of Defb2 and Defr1, the *in vitro* attractant activities of β-defensins appear to be more rigorous in that the attractant abilities are not inhibited by either serum or salt, whereas the antibacterial activities are (Zucht *et al.*, 1998). Furthermore, lower concentrations of defensins are required for attractant ability, which are more similar to the concentrations at which they have been detected in

tissues (Singh *et al.*, 1998; Ganz, 1998). Yang *et al.* have proposed a two-concentration model of defensin function (Yang *et al.*, 2000a). At nanomolar concentrations, defensins act as signalling molecules and chemoattract molecules as shown by this study and others (Yang *et al.*, 1999; Yang *et al.*, 2000a). Higher concentrations (in the micromolar range) of α- and β-defensins are required for antibacterial function, possibly because high concentrations permit the formation of higher order structures that may be required for antibacterial activity (Hoover *et al.*, 2000; Yang *et al.*, 2000a; Bauer *et al.*, 2001). These observations once again raise the question as to what are the functions of β-defensins *in vivo*.

4.3.6 Chemokinesis and Chemotaxis

Standard chemotaxis apparatus, such as the 48-well microchemotaxis chamber used here, do not permit the establishment of a concentration gradient. Consequently, therefore, it is not clear whether the migration reported here is chemotactic (a directed migration in the presence of a concentration gradient) or chemokinetic (non-directional movement in the absence of a gradient) and further studies are required to resolve this issue. Normally checkerboard assays are employed for this purpose (Pereira, 1997). In an initial study of cell migration, such as the ones reported here, only the bottom wells of the chemotaxis chamber are loaded with the attractant. However, in the checkerboard assay there is an increasing amount of attractant in the lower wells (as per usual); but there is also added an increasing amount of attractant in the upper wells, which contain the cells. Thus, in this system a point is reached where there is no longer a concentration difference between the upper and lower chambers and it is possible to discriminate between directional and non-directional movement.

A theoretical example is depicted in Table 4.1 in which number in bold represent true chemotaxis and those underlined indicate chemokinesis. Furthermore, a novel system for measuring chemotaxis that uses a series of microchannels to generate a chemical gradient of a chemoattractant has recently been described (Jeon *et al.*, 2002) and this system my prove to be useful in further investigations of chemokinetic/chemotactic migration.

[Defensin] in upper wells	Defensin concentration in lower wells				
	0	1	10	100	1000
0	25	**43**	**85**	**120**	**105**
1	26	30	**48**	79	90
10	28	29	68	85	91
100	27	30	39	53	93
1000	25	32	35	32	45

Table 4.3: Determination of chemotaxis by checkerboard assay. Various concentrations of defensin peptides are present in both the upper and lower wells and the numbers indicate the number of migrated cells per treatment. Numbers underline show chemokinesis, and numbers in bold demonstrate chemotaxis. Taken from Pereira, (1997).

4.3.7 Summary

It would be of great interest to investigate the range of attractant activity of these murine β-defensins. These studies have suggested that murine β-defensins Defb2 and Defr1 attract immature, but not mature, DCs, and also CD4+ T-cells. As was discussed above, further studies are required to investigate the whether the migratory activity observed in these studies is chemoattractant or chemokinetic. It would also be of interest to investigate which subset of T-cells Defb2 and Defr1 are acting upon. Furthermore, the effect of these murine β-defensins on CD8+ T-cell migration and on the migration and degranulation of mast cells are also worthy of further investigation.

Chapter 5: Expression of Human and Murine β-Defensins in Mammalian Cell Lines

5.1 Introduction

The data presented in Chapters 3 and 4 of this thesis analyses the antibacterial and chemoattractant activities of synthetic β-defensin peptides generated by Albachem Ltd. (Edinburgh, UK). Whilst such peptides have the advantages of convenience and purity and are ideal for *in vitro* analysis, their cost is very high and improper folding may compromise activity. Moreover, natural sources of β-defensins are also unsuitable as the levels at which β-defensins are produced in tissues are too low to be of use, for example in one study 136 μg of DEFB1 was purified form 10 litres of urine and 3.6 mg of DEFB1 from 10000 litres of haemofiltrate (Zucht *et al.*, 1998). The other main method of production therefore is to use recombinant DNA technology. This chapter describes an attempt to establish a mammalian cell line that has been stably transfected to express the β-defensins *DEFB1*, *DEFB4*, *Defb1* and *Defb2*.

The problems of cost and incorrect folding associated with the use of chemically synthesised peptides were introduced above. Further problems are highlighted by the identification of several different isoforms of human DEFB1, which were shown to have different spectra of antibacterial activity (Valore *et al.*, 1998). Valore *et al.* isolated several different forms of DEFB1 from urine that vary in length due to alternate splicing at the N-terminus. The peptides varied in length from 36 to 47 amino acids, with the 40- and 44-residue variants being the predominant forms. Interestingly, the recombinant

peptides generated using a baculovirus system were shown by subsequent *in vitro* analysis to have different levels of microbicidal activity. The 36-residue variant was the most potent and retained its activity under high salt conditions, whereas the other forms showed little or no activity in the presence of high levels of salt. Obviously, the synthetic peptides used in the studies described in this thesis are sequenced to a defined N-terminus. Therefore, they may not accurately reflect the natural β-defensin milieu, which may be composed of a cocktail of different isoforms varying at the NH_2-termini and each possessing different levels of antimicrobial activity. The establishment of a system producing recombinant protein may help to investigate this issue. Indeed, if a detailed analysis of this issue is required it may be necessary to transfect cells from different tissues, as the spectra of β-defensin N-terminus isoforms may vary among different tissues. However, the use of a baculovirus system in an insect cell line may not generate defensin variants that are relevant to mammalian cells, for example the pool of DEFB1 peptides generated by the baculovirus in the Valore *et al.* (1998) study differed from those isolated form urine in terms of which DEFB1 NH-terminus variant was predominant.

The potential for incorrect folding of synthetic β-defensin peptides is another issue associated with their use. For example, the structure of the synthetic Defr1 peptide used in the studies described in this thesis (Morrison *et al.*, 2002a) was analysed by Dr Perditta Barran (University of Edinburgh, UK) and was found to exist in both monomeric and dimeric forms. Moreover, as Defr1 lacks the first canonical cysteine the arrangement of the disulfide bridges was altered from the conserved arrangement found in all other β-defensins. The monomeric form of Defr1 contained three disulfide bridges but the dimeric form was revealed to be composed of two different forms,

differing in the cysteine-bonding pattern; one form contained four disulfide bonds and the other contained five. It is not clear whether these different structural forms are artefacts of the chemical synthesis or whether they represent naturally occurring isoforms. The identification and analysis of Defr1 peptide were made after the completion of the studies described in this chapter and therefore Defr1 is not included in the experiments described in this chapter. However, the issues associated with its structure illustrate well the need for recombinant production of peptides, and resolution of the Defr1 structural issues may benefit from analysis using such a system.

Several studies have used recombinant peptides produced using baculovirus system in an insect cell line (Bals *et al.*, 1998b; Singh *et al.*, 1998; Valore *et al.*, 1998). Both the Valore *et al.* and the Bals *et al.* studies report the production of human β-defensins, which vary in the sequence of the N-terminus. As described earlier Valore *et al.* report different levels of activity for the different recombinant DEFB1 peptide variants. Bals *et al.* (1998b) used a baculovirus system to generate recombinant DEFB4 (formerly DEFB2 or HBD2) peptide, they isolated two different isoforms that differ in length by three residues at the N-terminus, whereas Harder *et al.*, (1997) only isolated the shorter 38 amino acid form from the skin. However, these two forms did not differ in antimicrobial activity and it is not clear whether they represent an artefact of the insect cell line used, or if they represent genuine structural variants. In a study of Defb1, antimicrobial activity, Bals *et al.* (1998a) successfully produced functional recombinant peptide. A mammalian cell line was transiently transfected with a construct for Defb1, and the lysates of the transfected cell had significantly greater antimicrobial activity compared to the empty vector controls, however, this study did not purify the peptide.

This chapter describes an attempt to produce a stably transfected mammalian cell line expressing β-defensins. The establishment of a stably transfected cell line would add to the convenience of the system, in that β-defensins are constantly produced and samples can be taken as needed. Two different cell lines are used in this study, the human bronchial epithelial (HBE) cell line, which expresses *DEFB1* and *DEFB4*, and the mouse mammary epithelial C127 cell line, which does not express detectable levels of *Defb1* or *Defb2*. The system used in the studies presented in this chapter utilised the operator plasmid from the LacSwitch II system (Stratagene). Expression from the operator plasmid, pOPRSVI/MCS is driven by the Rous sarcoma virus promoter, and stable transfectants can be selected using geneticin (G418) resistance (Figure 5.1). There is also the option of using the repressor plasmid pCMVLacI, this is of use if the inserted gene is toxic as it prevents transcription until a suitable inducer is added (Figure 5.2). This plasmid uses the lactose (lac) repressor protein from the *Escherichia coli* lac operon; the lac repressor binds to the lac operator in the pOPRSVI/MCS vector and prevents transcription. When an inducer such as isopropyl β-D-thiogalactopyranoside (IPTG) is added to the culture media, it binds to the lac repressor causing a conformational change and reducing its affinity for the operator. When the repressor is removed from the operator, transcription of the inserted gene can occur.

In addition to the comparison of the *in vitro* antibacterial activity of recombinant peptides with the synthetic peptides, the production of recombinant β-defensins would permit further experiments that may otherwise prove to be impractical due to cost. Such experiments would be to investigate the antimicrobial activity of β-defensins in combination with each other and other aspects of the innate immune system such as lactoferrin and

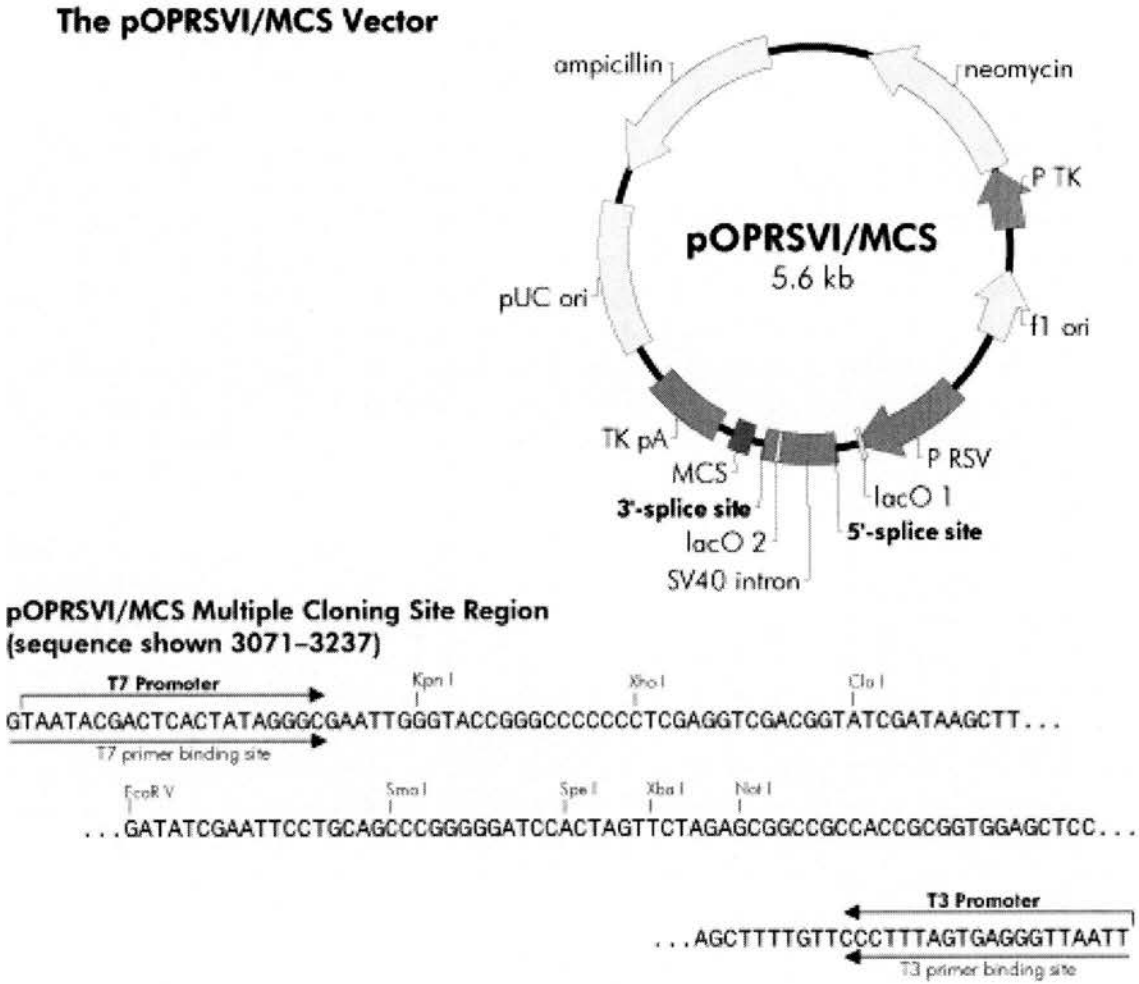

Figure 5.1: Structure multiple cloning site of operator plasmid pOPRSVI/MCS (Stratagene). β-Defensin cDNAs can be ligated into the vector using the multiple cloning site (MCS). Expression of the inserted gene from the expression plasmid can be regulated by co-transfection of target cells with the repressor plasmid (Figure 5.2).

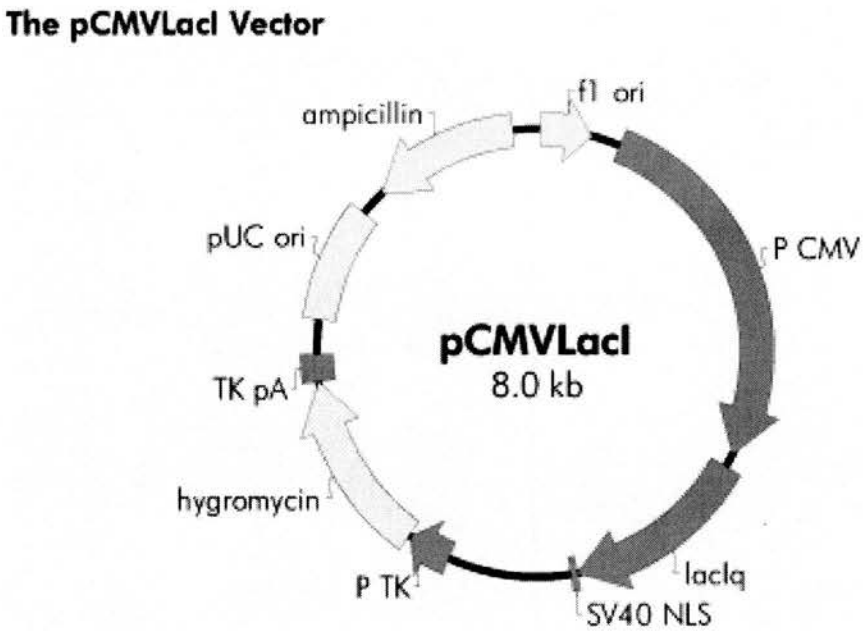

Figure 5.2: Structure of repressor plasmid pCMVLacI plasmid (Stratagene). This plasmid encodes the lac repressor, which will suppress expression of the of the β-defensin genes encoded in the expression plasmid (Figure 5.1). The lac repressor binds to the lac operator encoded on the expression plasmid. When IPTG is present in the culture media it binds to the lac repressor, causing a decrease in its affinity for the lac operator. When the repressor is removed expression of the inserted β-defensin gene can occur.

lysozyme, as previous studies have suggested that the activity of β-defensins is synergistic with such factors (Bals *et al.*, 1998b).

Production of recombinant peptides in a mammalian cell line may also permit the analysis of their native structure and N-terminal sequence, as with synthetic peptides the sequence, and to a certain extent, the structure is dictated by experimental conditions, and involves a certain degree of conjecture based on previously identified β-defensins. For example, the precise sequence of mature Defb2 is not known, however, the sequence of the form used in this study was predicted based on the reported sequence of mature Defb1, Defb3 and DEFB4. Using a stably transfected cell line expressing β-defensins may also permit the analysis of their antimicrobial activity on a cell monolayer. Unlike the *in vitro* assays that only measure the activity of a specific β-defensin in isolation, such an experiment would permit the analysis of the antibacterial activity of these peptides in conjunction with living epithelia and may represent a more physiologically relevant environment. For example, it may be possible to treat cultures of primary airway surface epithelial cells with the recombinant peptides and then expose the monolayer to bacteria to investigate the antibacterial activity of defensins in combination with factors produced by epithelial cells such as lactoferrin and lysozyme.

5.2: Results

5.2.1 Cloning of complementary DNAs for *DEFB1* and *DEFB4*

A human bronchial epithelial (HBE) cell line was treated with 100 μg of *Escherichia coli* Lipopolysaccharide (LPS) for 4 or 24 hrs; RNA was isolated from these cell lines and an untreated control. Complementary DNA (cDNA) was synthesised from the isolated RNA and used in reverse-transcriptase polymerase chain reaction (RT-PCR) reactions to isolate cDNAs for *DEFB1* and *DEFB4* (Figure 5.2). *DEFB1* was detected at all time points, but expression of *DEFB4* was only in detected in those cell lines pretreated with LPS. The PCR products were ligated into a pCR II-TOPO cloning vector to give *DEFB1*/pTOP-TA and *DEFB4*/pCR II-TOPO constructs.

Plasmid DNA was isolated from bacteria transformed with the *DEFB-1*/TOP-TA or *DEFB4*/pTOPO-TA constructs and the defensin inserts were sequenced using the Sp6 and T7 plasmid-contained primer sites to confirm the fidelity of the PCR (Figure 5.3 and 5.4). Inserts that showed 100% identity to the *DEFB1* or *DEFB4* cDNA sequence were selected for further work.

DEFB1 was excised from the pCR II-TOPO vector by the Spe I (5′) and Not I (3′) restriction endonuclease sites and subcloned in to the pOPRSVI/MCS vector to give *DEFB1*/pOPRSVI. DEFB4 was excised by the Eco RV (5′) and Spe I (3′) site and also ligated in to the pOPRSVI/MCS vector. Plasmid DNA was isolated from transformed bacteria, and the inserts sequenced using the KS and T3 (DEFB1) and T3 and T7 (DEFB4) plasmid-contained primer sites to confirm the fidelity of the cDNA insert.

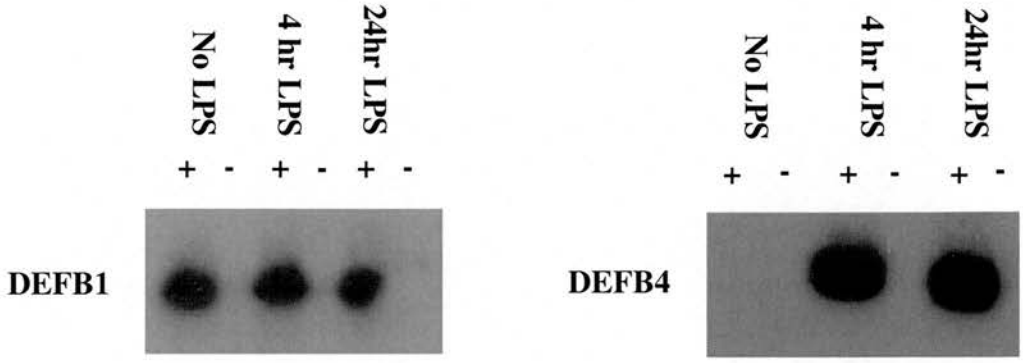

Figure 5.3: Cloning of *DEFB1* and *DEFB4* from human bronchial epithelial cell line.

Human bronchial epithelial cells were treated with 100 μg LPS, RNA was isolated at 4 and 24 hrs post-treatment and used in RT-PCR analysis for expression of DEFB1 and DEFB4. The generated PCR products were cloned into pTOPO-TA vector and sequenced to confirm their identity.

A) ***DEFB1***

```
12  TCCAAAGGAGCCAGCCTCTCCCCAGTTCCTGAAATCCTGAGTGTTGCCTG
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    tccaaaggagccagcctctccccagttcctgaaatcctgagtgttgcctg
              .         .         .         .         .
62  CCAGTCGCCATGAGAACTTCCTACCTTCTGCTGTTTACTCTCTGCTTACT
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    ccagtcgccatgagaacttcctaccttctgctgtttactctctgcttact
              .         .         .         .         .
112 TTTGTCTGAGATGGCCTCAGGTGGTAACTTTCTCACAGGCCTTGGCCACA
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    tttgtctgagatggcctcaggtggtaactttctcacaggccttggccaca
              .         .         .         .         .
162 GATCTGATCATTACAATTGCGTCAGCAGTGGAGGGCAATGTCTCTATTCT
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    gatctgatcattacaattgcgtcagcagtggagggcaatgtctctattct
              .         .         .         .         .
212 GCCTGCCCGATCTTTACCAAAATTCAAGGCACCTGTTACAGAGGGAAGGC
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    gcctgcccgatctttaccaaaattcaaggcacctgttacagagggaaggc
              .         .         .         .         .
262 CAAGTGCTGCAAGTGAGCTGGGAGTGACCAGAAGAAATGACGCAGAAGTG
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    caagtgctgcaagtgagctgggagtgaccagaagaaatgacgcagaagtg
              .
312 AAATGAACTTTTT
    |||||||||||||
    aaatgaacttttt
```

B) ***DEFB4***

```
11  CCAGCCATCAGCCATGAGGGTCTTGTATCTCCTCTTCTCGTTCCTCTTCA 60
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    ccagccatcagccatgagggtcttgtatctcctcttctcgttcctcttca
              .         .         .         .         .
61  TATTCCTGATGCCTCTTCCAGGTGTTTTTGGTGGTATAGGCGATCCTGTT 110
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    tattcctgatgcctcttccaggtgttttggtggtataggcgatcctgtt
              .         .         .         .         .
111 ACCTGCCTTAAGAGTGGAGCCATATGTCATCCAGTCTTTTGCCCTAGAAG 160
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    acctgccttaagagtggagccatatgtcatccagtcttttgccctagaag
              .         .         .         .         .
161 GTATAAACAAATTGGCACCTGTGGTCTCCCTGGAACAAAATGCTGCAAAA 210
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    gtataaacaaattggcacctgtggtctccctggaacaaaatgctgcaaaa
              .         .         .         .         .
211 AGCCATGAGGAGGCCAAGAAGCTGCTGTGGCTGATGCGGATTCAGAAAGG 260
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    agccatgaggaggccaagaagctgctgtggctgatgcggattcagaaagg

261 GCTCC 265
    |||||
    gctcc
```

Figure 5.4: Sequencing of (A) *DEFB1* and (B) *DEFB4* cloned by RT-PCR and ligated into the pTOPO-TA vector. Comparison to previously PCR products (in lowercase) identified sequences (in uppercase lettering) was performed using Bestfit function in GCG (www.hgmp.mrc.ac.uk).

A) ***Defb1***

```
 17 CACTCTGGACCCTGGCTGCCACCACTATGAAAACTCATTACTTTCTCCTG 66
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    cactctggaccctggctgccaccactatgaaaactcattactttctcctg
             .         .         .         .         .
 67 GTGATGATATGTTTTCTTTTCTCCCAGATGGAGCCAGGTGTTGGCATTCT 116
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    gtgatgatatgttttcttttctcccagatggagccaggtgttggcattct
             .         .         .         .         .
117 CACAAGTCTTGGACGAAGAACAGATCAATACAAATGCCTTCAACATGGAG 166
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    cacaagtcttggacgaagaacagatcaatacaaatgccttcaacatggag
             .         .         .         .         .
167 GATTCTGTCTCCGCTCCAGCTGCCCATCTAATACCAAACTACAGGGAACC 216
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    gattctgtctccgctccagctgcccatctaataccaaactacagggaacc
             .         .         .         .         .
217 TGTAAACCAGATAAGCCCAACTGTTGTAAGAGCTGACAGTAGTTTGAAGA 266
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    tgtaaaccagataagcccaactgttgtaagagctgacagtagtttgaaga
             .         .
267 ATGGACATAAAGGACGAGCGATGGATT 293
    |||||||||||||||||||||||||||
    atggacataaaggacgagcgatggatt
```

B) ***Defb2***

```
  1 CTCTCTGGAGTCTGAGTGCCCTTTCTACCAGCCATGAGGACTCTCTGCTC
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    ctctctggagtctgagtgcccttctaccagccatgaggactctctgctc
             .         .         .         .         .
 51 TCTGCTGCTGATATGCTGCCTCCTTTTCTCATATACCACTCCAGCTGTTG
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    tctgctgctgatatgctgcctcctttctcatataccactccagctgttg
             .         .         .         .         .
101 GAAGTTTAAAAAGTATTGGATACGAAGCAgAACTTGACCACTGCCACACC
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    gaagtttaaaaagtattggatacgaagcagaacttgaccactgccacacc
             .         .         .         .         .
151 AATGGAGGGTACTGTGTCAGAGCCATTTGTCCTCCTTCTGCCAGGCGTCC
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    aatggagggtactgtgtcagagccatttgtcctccttctgccaggcgtcc
             .         .         .         .         .
201 TGGGAGCTGTTTCCCAGAGAAGAACCCCTGTTGCAAGTACATGAAATGAT
    ||||||||||||||||||||||||||||||||||||||||||||||||||
    tgggagctgtttcccagagaagaaccccctgttgcaagtacatgaaatgat
             .         .         .
251 TAGAAGGAAGCACATGGAAGTCAAGTGACA
    ||||||||||||||||||||||||||||||
    tagaaggaagcacatggaagtcaagtgaca
```

Figure 5.5: Sequencing of (A) *Defb1* and (B) *Defb2* previously cloned by RT-PCR and ligated into the pTOPO-TA vector. Comparison to previously PCR products (in lowercase) identified sequences (in uppercase lettering) was performed using Bestfit function in GCG (www.hgmp.mrc.ac.uk).

DNAs for *Defb1* and *Defb2* had been previously isolated from mouse tissues and ligated into the pOPRSVI/MCS vector (*Defb1*/pOPRSVI and *Defb2*/pOPRSVI) by Gillian Morrison working in this laboratory and these constructs were also sequenced using T3 and KS primer sites (Figure 5.5). Inserts that showed 100% identity to the appropriate defensin cDNA sequence were used for later studies.

5.2.2 Transfection of mammalian cell lines with defensin-pOPRSVI constructs

Mouse mammary epithelial C127 cell lines and human HBE cell lines were transfected with *Defb1*/pOPRSVI, *Defb2*/pOPRSVI, *DEFB1*/pOPRSVI, or *DEFB4*/pOPRSVI using the calcium phosphate method as described in material and methods. The cell lines were treated with 500 μg/ml Geneticin (G418) to select for stably transfected cell lines. After 14-21 days several G418-resistant clones were visible. Single colonies and groups of colonies were picked and cultured separately with maintenance of selection. In total, from two separate transfection experiments, 13 colonies were isolated from C127 cells transfected with the DEFB1/pOPRSVI construct, 19 colonies from cells transfected with the DEFB2/pOPRSVI construct, eleven from cells transfected with Defb1/pOPRSVI and 21 from Defb2/pOPRSVI transfected cells. Attempts were also made to establish stable cell lines transfected with the repressor plasmid pCMVLacI, which can they be cultured and transfected with the defensin/pOPRSVI constructs. However, after several attempts no stable clones were identified.

Attempts were made to establish stable cell lines transfected with the repressor plasmid pCMV/LacI, whiuch could then be cultured and

transfected with the defensin/pOPRSVI constructs. However, after several attempts no stable clones were identified. Furthermore, repeated attempts to transfect the HBE cell line with the defensin/pOPRSVI constructs also failed to generate any G418-resistant clones.

5.2.3 Analysis of expression of defensins by transfected C127 cell lines

RT-PCR analysis of several of the β-defensin/pOPRSVI transfected clones was conducted to identify those clones expressing the transfected defensin. Untransfected C127 cell lines do not express detectable levels of *Defb1* or *Defb2*, however, all transfected-C127 cell lines analysed expressed the appropriate defensin (Figure 5.5). Eight clones were identified that express *Defb1*, 13 clones expressed detectable levels of *Defb2*, ten that expressed DEFB1 and eleven that expressed DEFB4. The reproducibility and validity of the expression was tested by repeating the RT-PCR three times (i.e. n=3) using separate samples of freshly RNA.

Twelve G418-resistant clones expressing the transfected-defensins (three for each of the transfected defensin-plasmid constructs) were selected for further analysis. Further RT-PCR analyses showed that these cell lines expressed only the defensin with which that cell line had been transfected, expression of the other β-defensins was not detected (Figure 5.6).

5.2.4 Assessment of the antibacterial activity of transfected cell lines – Microbicidal activity of culture media

In order to investigate if the cell lines expressing β-defensins exhibit increased antibacterial activity the microbicidal effect of cell culture media (RPMI + 2.5% foetal calf serum) against *Pseudomonas aeruginosa* laboratory strain PAO1 and *Staphylococcus aureus* cystic fibrosis (CF) isolate C1705 was assessed. The antibacterial activity was assessed by incubating 2.5 x 10^4 colony-forming units (cfu) in 200 µl of culture media and incubating at 37°C for 30 min. All experimental results were repeated three times to test for reproducibility, using different samples of media each time (i.e. n=3).

Culture media from the β-defensin expressing cell lines did not show increased or reproducible antibacterial activity against either *P. aeruginosa* PAO1 or *S. aureus* C1705 compared to the untransfected or empty vector controls (Figure 5.9A). These experiments were repeated with culture media that had been dialysed against dH_2O to remove inhibitory effects of salt, which may inhibit antibacterial effects of the β-defensins. However, again the transfected cell lines did not demonstrate significantly greater antibacterial activity compared to the controls (Figure 5.9B). The above experiments were also repeated with $1x10^3$ cfu; however, significant numbers of surviving bacteria were isolated from these experiments (data not shown).

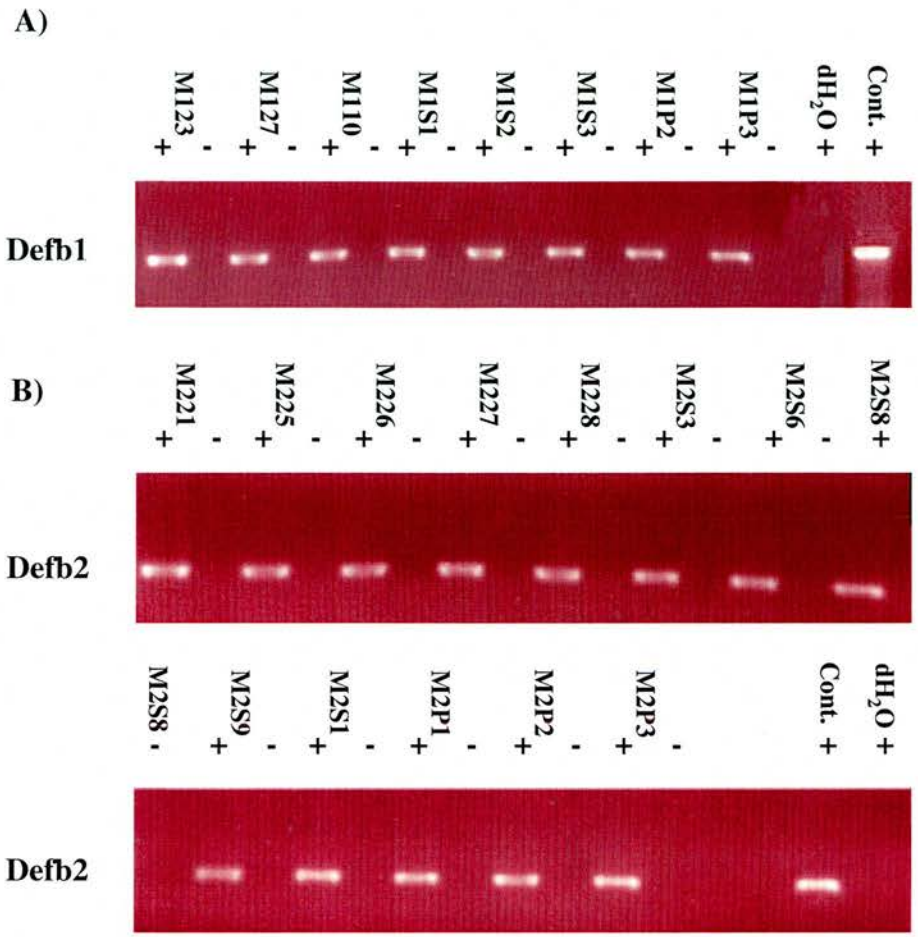

Figure 5.6: RT-PCR Analysis of β-Defensin Transfected C127 Cell Lines

A) Shows results of RT-PCR on RNA from C127 cells transfected with *Defb1*-containing vector, B) shows RT-PCR of RNA from *Defb2*/POPRSVI transfected C127 cells.

RNA was isolated from G418-resistant C127 cell lines that had been transfected with the β-defensin/POPRSVI constructs, and used for RT-PCR analysis as described in materials and methods. 100 ng of vector containing the appropriate β-defensin cDNA was used as a positive control. Reactions without reverse-transcriptase were set up as an internal control. Letter and number in the top row represent the name of the β-defensin transfected cell line. M1= Defb1 and M2= Defb2, Subsequent letters represent the number of the colony at initial subculture. RT-PCR analysis was repeated three times, using a new sample of freshly isolated RNA each time, to ensure the validity of the result (n=3).

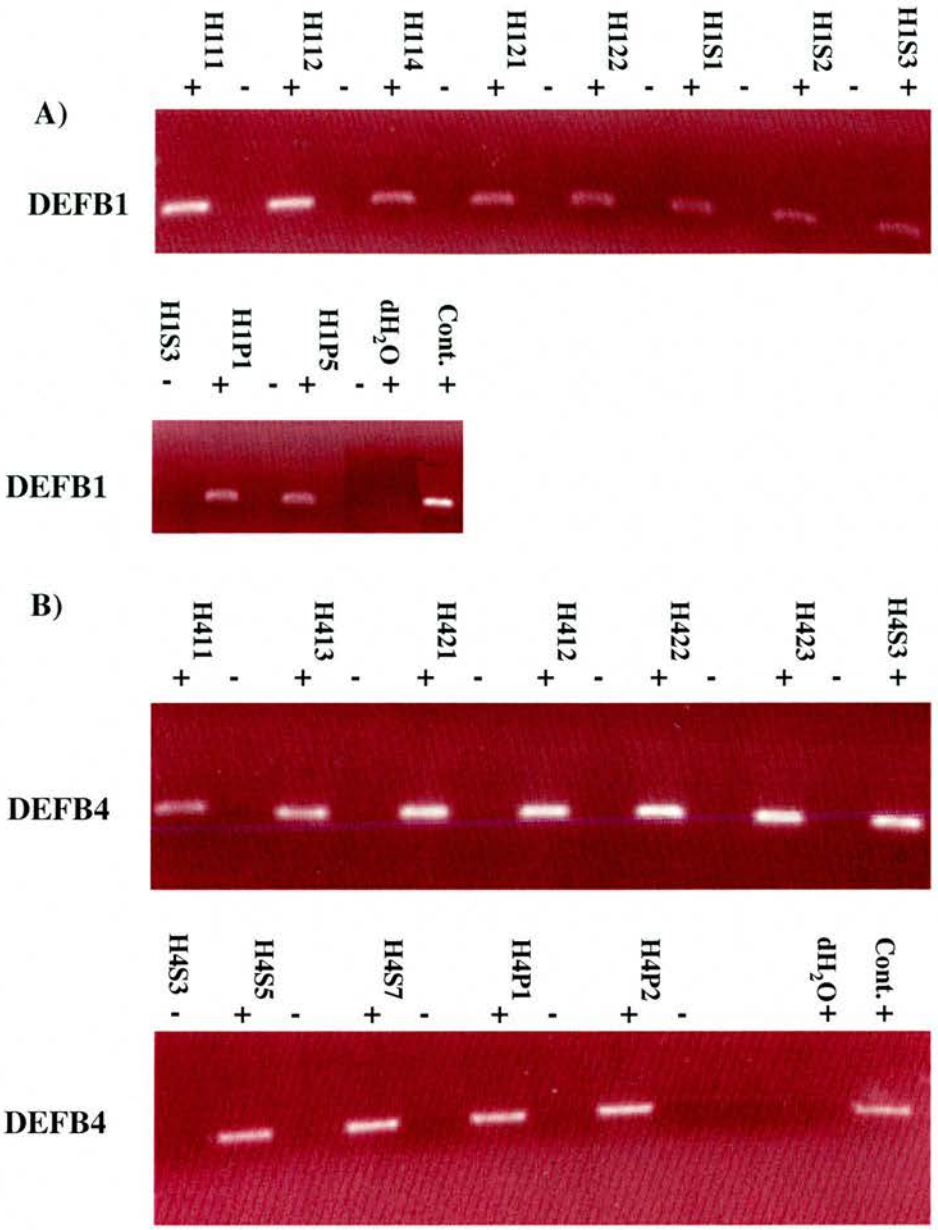

Figure 5.7 : RT-PCR Analysis of β-Defensin Transfected C127 Cell Lines

A) shows RT-PCR analysis of cells transfected with *DEFB1*/pOPRSVI construct and B) represents expression analysis of cells transfected with the *DEFB4*/pOPRSVI plamsid.

RNA was isolated form G418-resistant C127 cell lines that had been transfected with the β-defensin/POPRSVI constructs, and used for RT-PCR analysis as described in materials and methods. 100 ng of vector containing the appropriate β-defensin cDNA was used as a positive control. Reactions without reverse-transcriptase were setup as an internal control. Letter and number in the top row represent the name of the β-defensin transfected cell line. H1=DEFB1 and H2=DEFB4. Subsequent letters represent the number of the colony at initial subculture. RT-PCR analysis was repeated three times, using a new sample of freshly isolated RNA each time, to ensure the validity of the result (n=3).

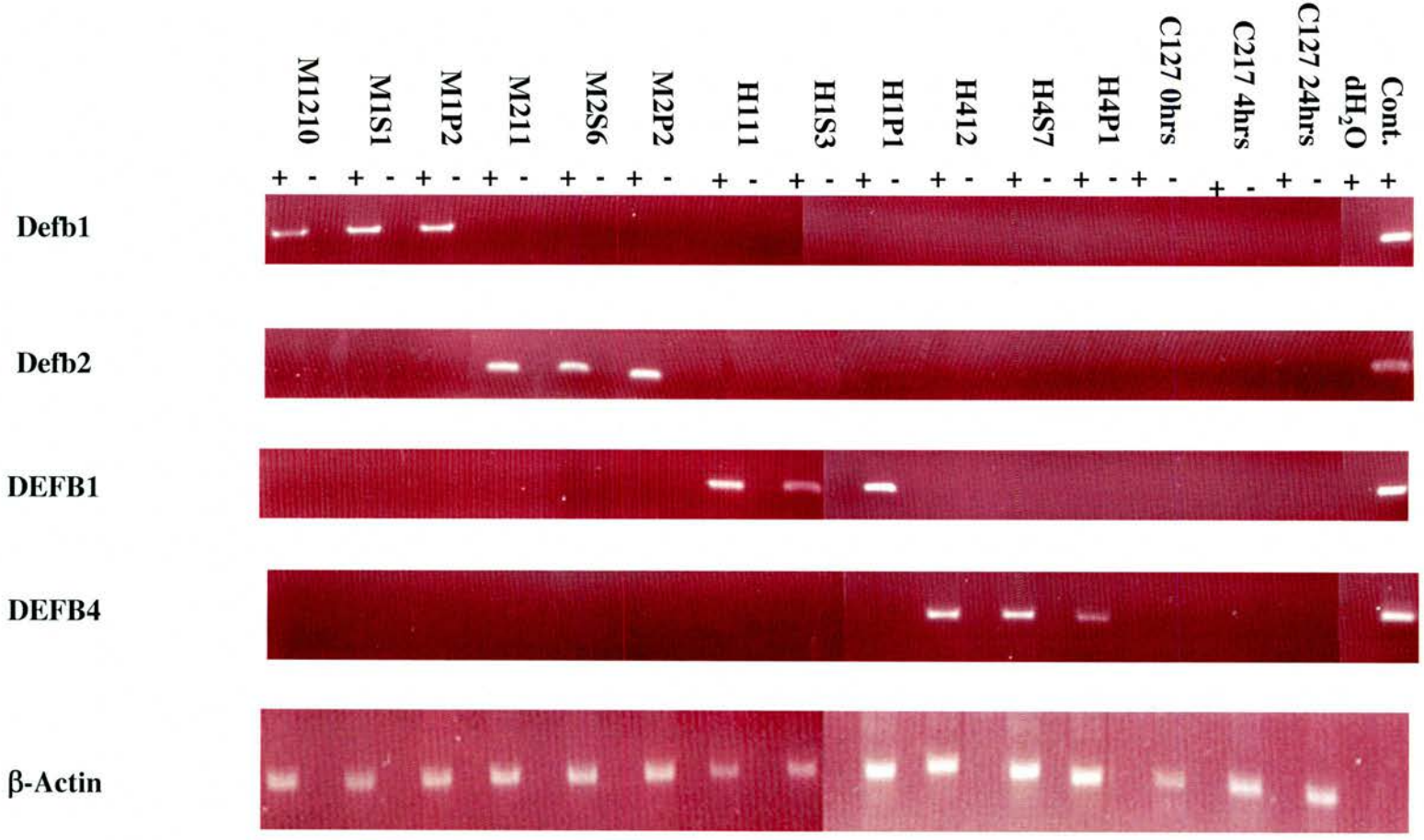

Figure 5.8: RT-PCR analysis of Expression of β-Defensins by transfected C127 Cell Lines RT-PCR Analysis was performed as described in materials and methods. 100 ng of vector containing the appropriate β-defensin cDNA was used as a positive control, reactions were set up without reverse-transcriptase as controls and β-actin RT-PCR was performed to confirm integrity of RNA samples. Letter and number in the top row represent the name of the β-defensin transfected cell line. M1= Defb1, M2= Defb2, H1=DEFB1, H2=DEFB4. Subsequent letters represent the number of the colony at initial subculture. RT-PCR analysis was repeated three times, using a new sample of freshly isolated RNA each time, to ensure the validity of the result (n=3).

5.2.5 Assessment of the antibacterial activity of transfected cell lines – Microbicidal activity of cell lysates

The antibacterial activity of cell lysates was also assessed as functional β-defensin peptides may have been retained within the cells and not secreted into the culture media. Cell pellets from a 150cm^2 culture dish were lysed by brief sonication, and the antibacterial activity was assessed by incubating 1 x 10^4 cfu with the lysed cell pellet in 200 µl of phosphate buffer.

No significant reproducible difference was observed between the antibacterial activity of cell pellets from untransfected or transfected cell line against *P. aeruginosa* PAO1 or *S. aureus* C1705 (Figure 5.9C). Dialysed cell lysates form β-defensin transfected cell lines also failed to demonstrate significantly increased antibacterial activity compared to the controls (Figure 5.9D). As above these experiments were repeated using 1 x10^3 cfu, but again significant numbers of bacteria were not isolated from this experiment to permit valid assessment. All experimental results were repeated three times to test for reproducibility, using different samples of cell lysate each time (i.e. n=3).

5.2.6 Assessment of the antibacterial activity of transfected cell lines – Microbicidal activity of purified culture media and cell lysates

Experiments described above have failed to demonstrate increased antimicrobial activity of culture media or cell lysates from cells expressing the β-defensins *DEFB1*, *DEFB4*, *Defb1* or *Defb2* compared to untransfected or

empty vector controls. Therefore, attempts were made to purify cationic peptides from lysates and culture media following a protocol established by Dr Gerry McLachlan (University of Edinburgh, UK) based on that of Diamond *et al.* (1991). The lysates from ten cell pellets of the various β-defensin-expressing cell lines were mixed with 45 ml of media isolated from the same cell line were purified using a C_{18} Sep-Pak cartridge as described in the Materials and Methods. The antibacterial assays described above were repeated using the purified lysates and media. However, as in previous studies no significant difference was observed between the antibacterial activities of β-defensin expressing cell lines compared to that of the controls (Figure 5.9E). Experiments were repeated three times to ensure reproducibility three times (*i.e.* n=3).

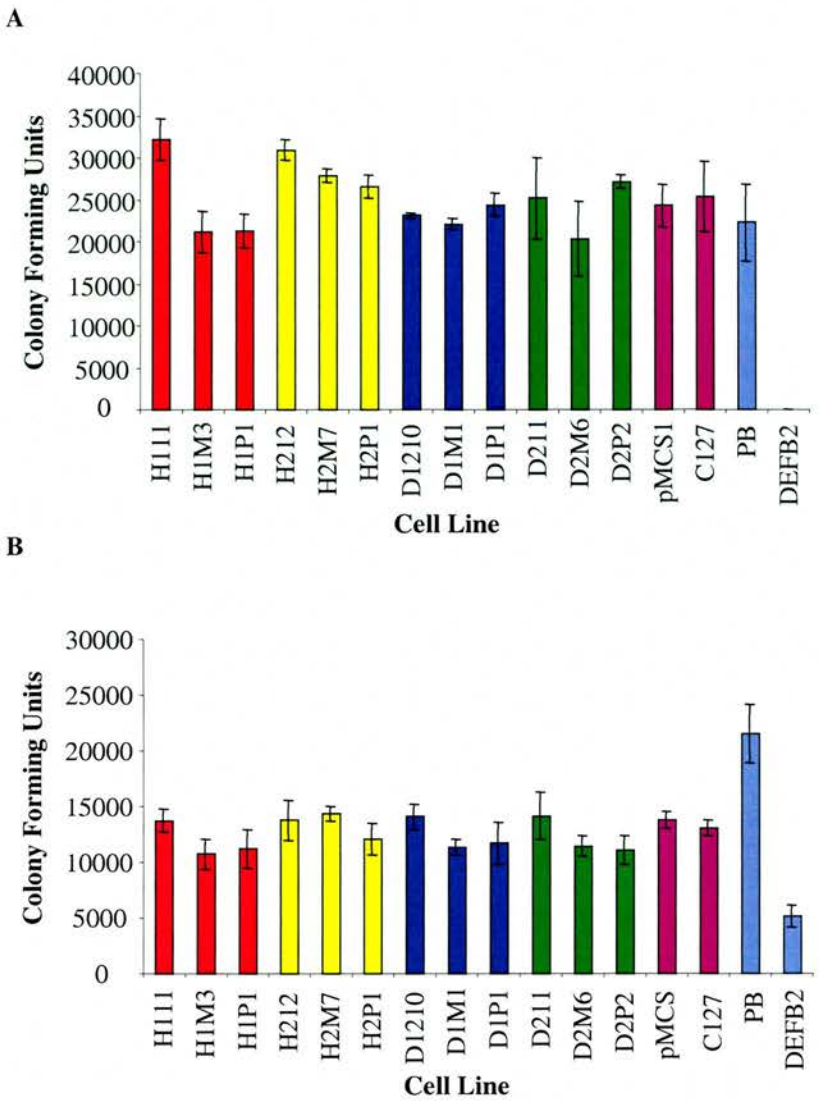

Figure 5.9: Antibacterial Activity of Culture Media from β-defensin transfected C127 Cell Lines.

Graph A shows antibacterial activity of media incubated with *P. aeruginosa* PAO1. Graph B shows antibacterial activity of media against *S. aureus* C1705. 2.5 x 10^4 cfu were added to culture media and incubated for 30 min. The surviving were bacteria plated out, incubated overnight at 37°C and the resultant colonies were counted. Letter and number on the x-axis represent the name of the β-defensin transfected cell line. M1= Defb1, M2= Defb2, H1=DEFB1, H2=DEFB4. Subsequent letters represent the number of the colony at initial subculture. Antibacterial experiments were repeated three time, using a new sample of freshly isolated media each time, to ensure the validity of the result (n=3).

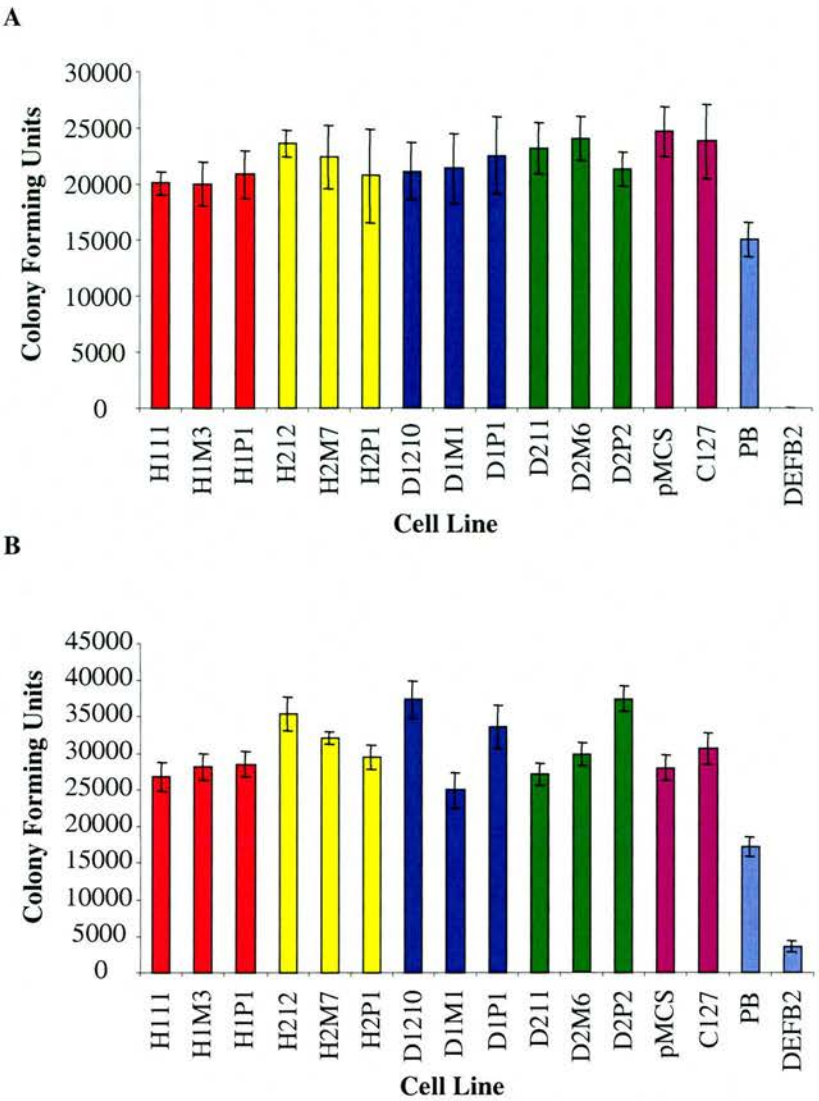

Figure 5.10: Antibacterial Activity of Dialysed Culture Media from β-defensin transfected C127 Cell Lines.

Graph A shows antibacterial activity of media incubated with *P. aeruginosa* PAO1. Graph B shows antibacterial activity of media against *S. aureus* C1705. 2.5 x 10^4 cfu were added to culture media and incubated for 30 min. The surviving were bacteria plated out, incubated overnight at 37°C and the resultant colonies were counted. Letter and number on the x-axis represent the name of the β-defensin transfected cell line. M1= Defb1, M2= Defb2, H1=DEFB1, H2=DEFB4. Subsequent letters represent the number of the colony at initial subculture. Antibacterial experiments were repeated three time, using a new sample of freshly isolated media each time, to ensure the validity of the result (n=3).

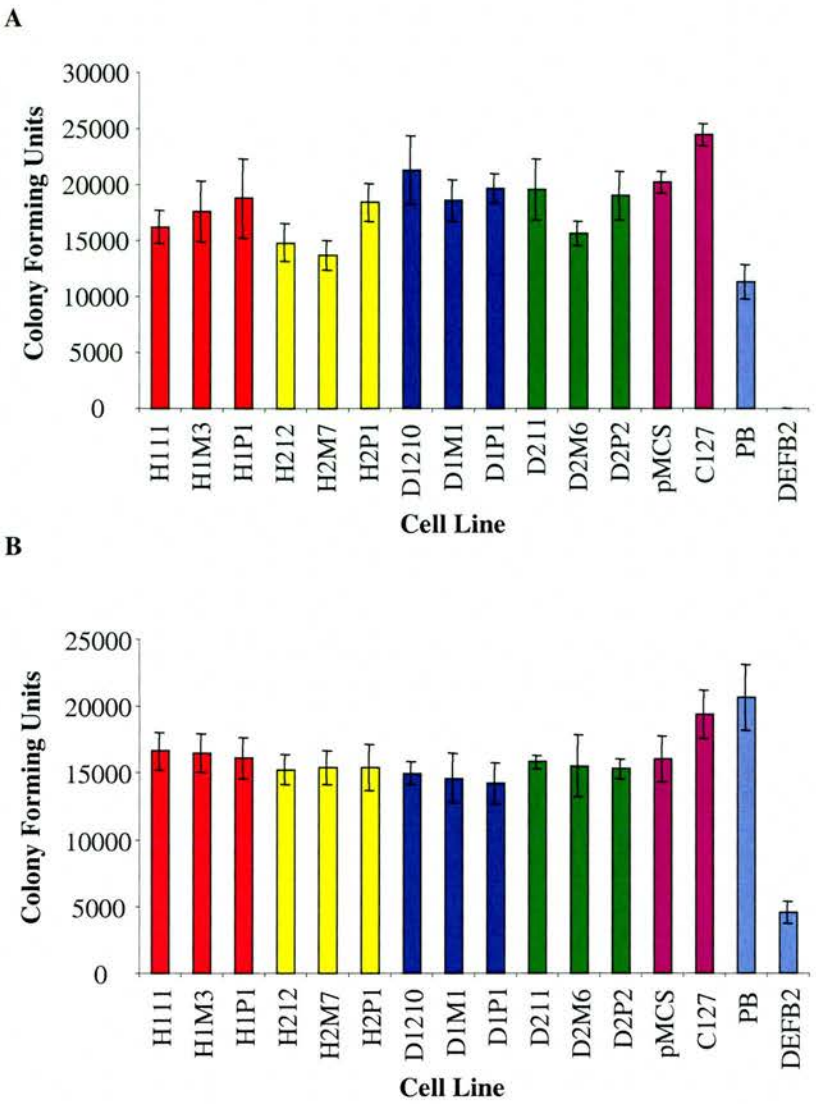

Figure 5.11: Antibacterial Activity of Lysates from β-defensin transfected C127 Cell Lines.

Graph A shows antibacterial activity of media incubated with *P. aeruginosa* PAO1. Graph B shows antibacterial activity of media against *S. aureus* C1705. 2.5 x 10^4 cfu were added to culture media and incubated for 30 min. The surviving were bacteria plated out, incubated overnight at 37°C and the resultant colonies were counted. Letter and number on the x-axis represent the name of the β-defensin transfected cell line. M1= Defb1, M2= Defb2, H1=DEFB1, H2=DEFB4. Subsequent letters represent the number of the colony at initial subculture. Antibacterial experiments were repeated three time, using a new sample of freshly isolated cell lysate each time, to ensure the validity of the result (n=3).

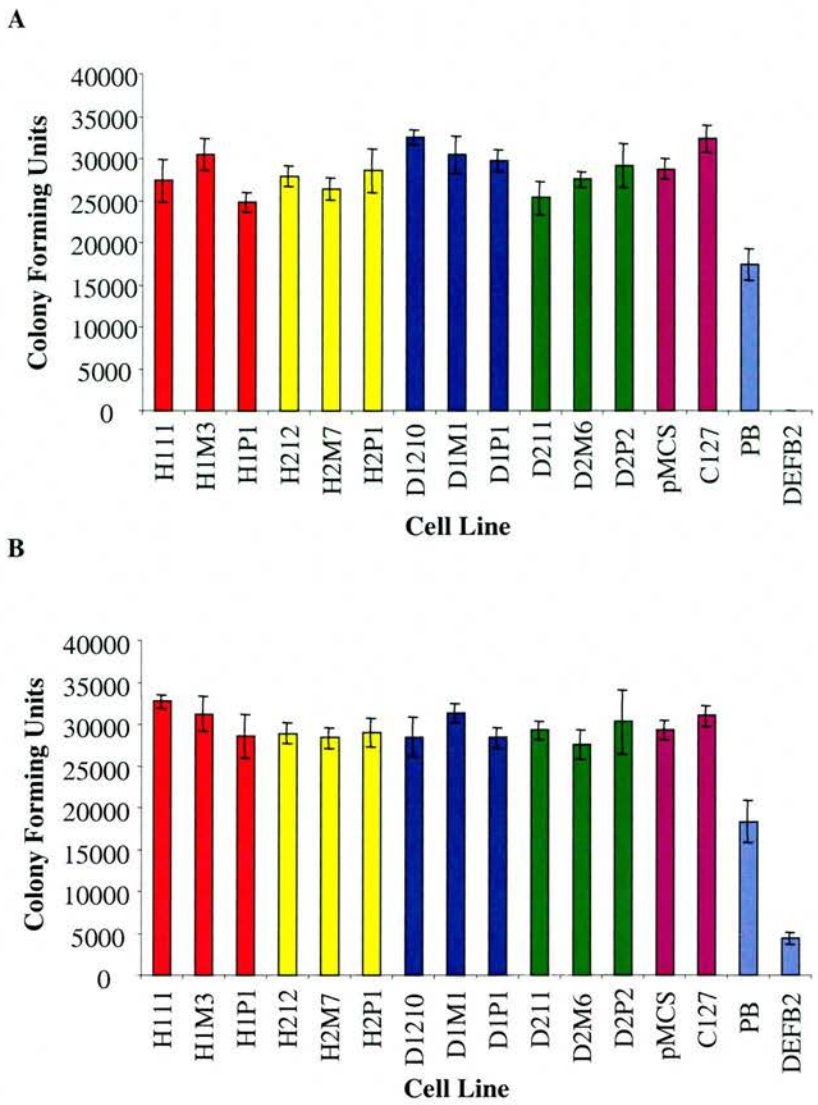

Figure 5.12: Antibacterial Activity of Dialysed Lysates from β-defensin transfected C127 Cell Lines.

Graph A shows antibacterial activity of media incubated with *P. aeruginosa* PAO1. Graph B shows antibacterial activity of media against *S. aureus* C1705. 2.5 x 10^4 cfu were added to culture media and incubated for 30 min. The surviving were bacteria plated out, incubated overnight at 37°C and the resultant colonies were counted. Letter and number on the x-axis represent the name of the β-defensin transfected cell line. M1= Defb1, M2= Defb2, H1=DEFB1, H2=DEFB4. Subsequent letters represent the number of the colony at initial subculture. Antibacterial experiments were repeated three time, using a new sample of freshly isolated cell lysate each time, to ensure the validity of the result (n=3).

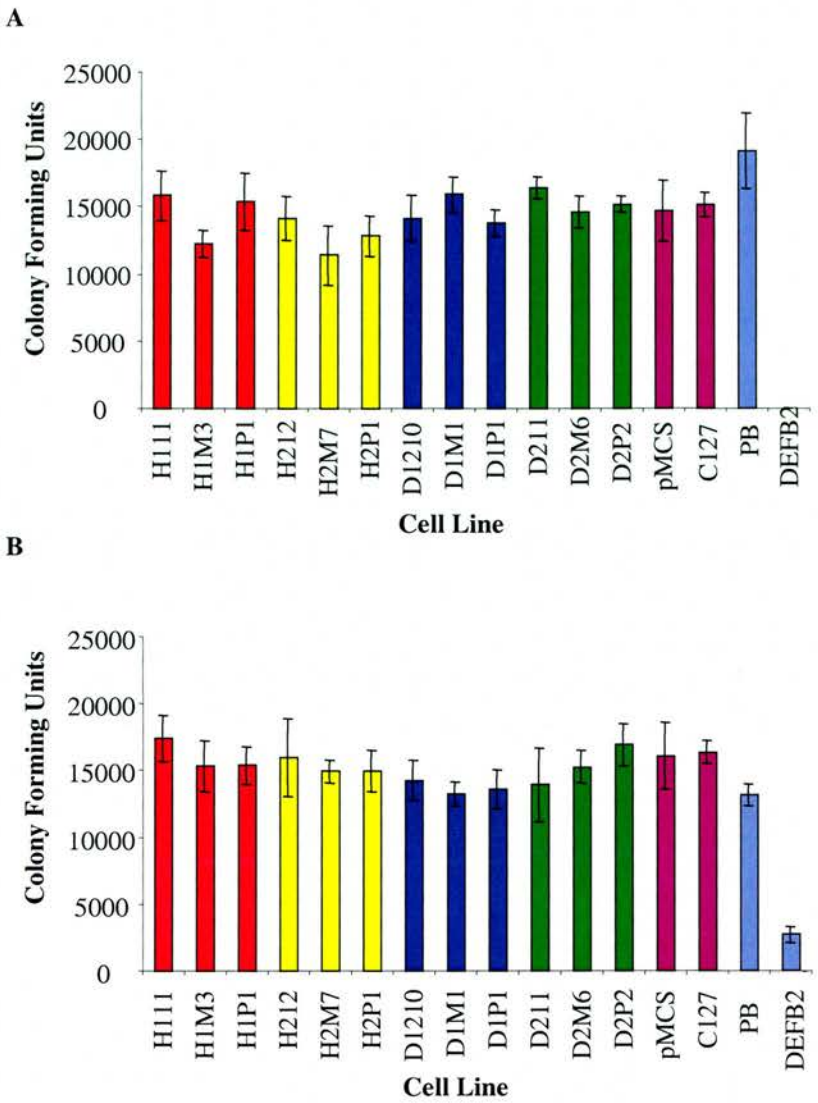

Figure 5.13: Antibacterial Activity of Purified Lyates/Culture Media from β-defensin transfected C127 Cell Lines.

Graph A shows antibacterial activity of media incubated with *P. aeruginosa* PAO1. Graph B shows antibacterial activity of media against *S. aureus* C1705. 2.5 x 10^4 cfu were added to culture media and incubated for 30 min. The surviving were bacteria plated out, incubated overnight at 37°C and the resultant colonies were counted. Letter and number on the x-axis represent the name of the β-defensin transfected cell line. M1= Defb1, M2= Defb2, H1=DEFB1, H2=DEFB4. Subsequent letters represent the number of the colony at initial subculture. Antibacterial experiments were repeated three time, using a new sample of freshly purified batch of media/cell lysate each time, to ensure the validity of the result (n=3).

5.2.7 Northern blot analysis of β-defensin expression by transfected cell lines

The expression of β-defensin mRNA by the transfected C127 cell lines was also analysed by northern blot. However, expression appropriate transfected β-defensin could not be detected in any of the cell lines analysed (Figure 5.8). A signal was detected in the positive control sample, which was 10 ng of the appropriate defensin cDNA, demonstrating the integrity of the β-defensin probe. Moreover, expression of β-actin was detected in all the cell lines, thus confirming the integrity of the mRNA. Northern analysis β-defensin expression by transfected cell lines was repeated twice, using different samples of freshly isolated RNA, to ensure reproducibility of the result.

5.2.8 Protein Gel analysis of β-defensin expression by transfected cell lines

Culture media, cell lysates, and purified media/lysates form all cell lines were separated on protein gels to investigate if β-defensin expressing cell lines possessed additional protein bands. However, no additional protein bands were visible in the β-defensin transfected cell lines compared to the untransfected or empty vector controls (Figure 5.9 and data not shown). Gel analysis of the protein content was repeated twice using fresh protein samples each time, to ensure the validity of the result.

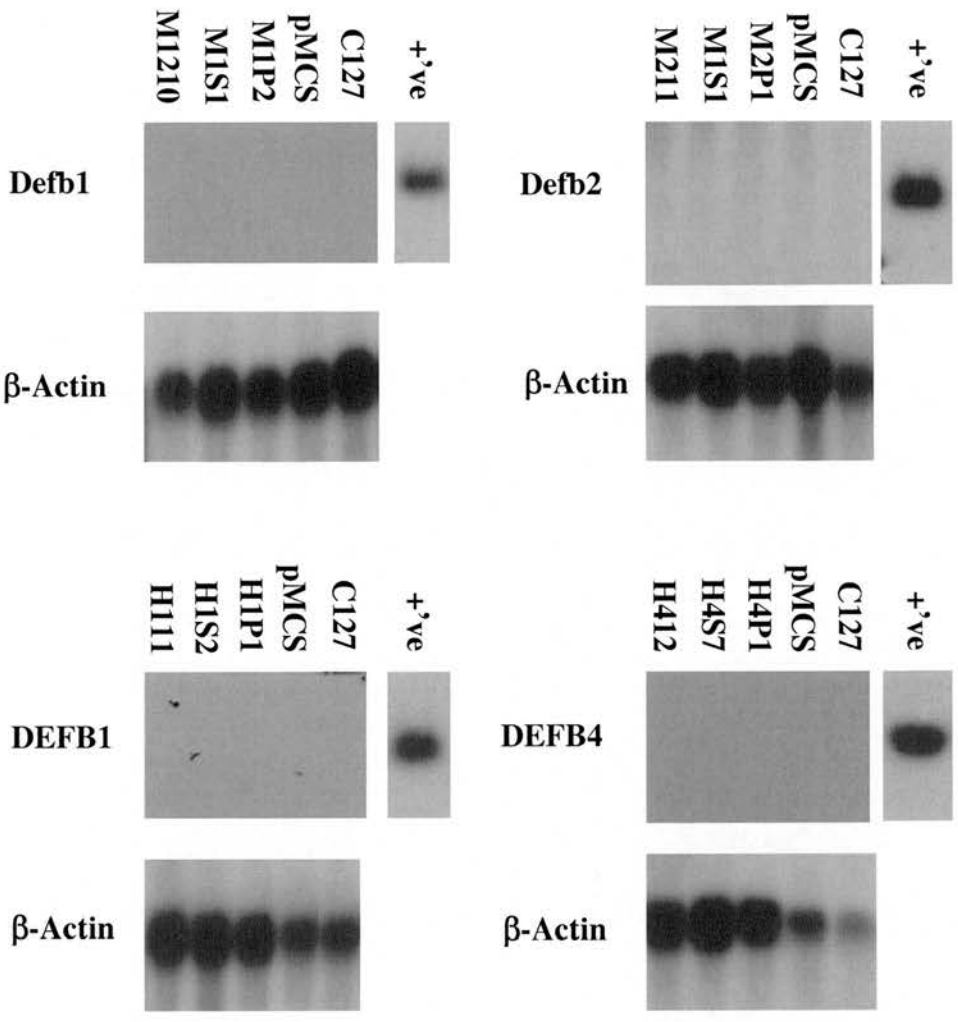

Figure 5.14: Northern Blot Analysis of β-Defensin Expression by Transfected C127 Cell Lines.

RNA was isolated from the different transfected cell lines; 20 μg was run on a denaturing gel and subjected to Northern blot analysis using the appropriate β-defensin cDNA as a probe. 10 ng of the appropriate β-defensin cDNA was run in parallel as a positive control for hybridisation. pMCS is the empty vector control, and C127 the untransfected cell line. Letter and number in the top row represent the name of the β-defensin transfected cell line. M1= Defb1, M2= Defb2, H1=DEFB1, H2=DEFB4. Subsequent letters represent the number of the colony at initial subculture. Northern blot analysis was repeated twice, using a new sample of freshly isolated RNA each time, to ensure the validity of the result (n=2).

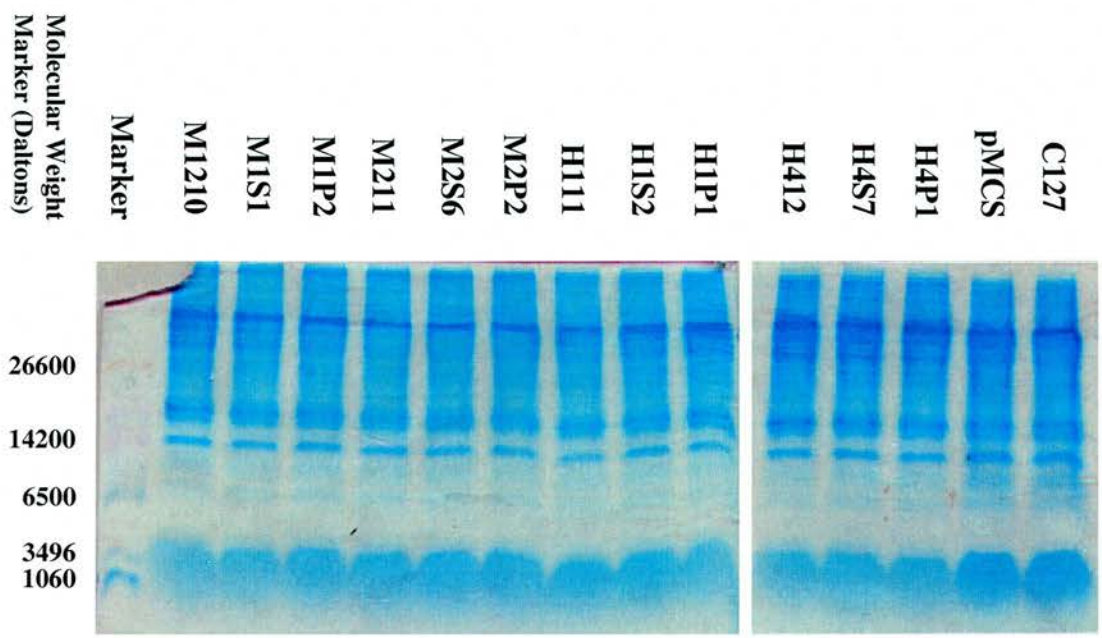

Figure 5.15: Protein Gel Analysis of the Protein Content of Culture Media Harvested from β-Defensin Expressing Cell Lines.

Culture media was harvested form β-defensin-transfected cell lines, boiled for 10 min with sample buffer and then separated on a Tris-tricine gel. Letter and number in the top row represent the name of the β-defensin transfected cell line. M1= Defb1, M2= Defb2, H1=DEFB1, H2=DEFB4. Subsequent letters represent the number of the colony at initial subculture. Protein gel analysis was repeated twice, using a new protein sample each time, to ensure the validity of the result (n=2). Similar analysis were performed on cell lysates and purified media/lysates (data not shown).

Chapter 5.3: Discussion

This chapter describes the establishment and characterisation of mouse mammary epithelial C127 cell lines stably transfected with murine and human β-defensins. However, whilst expression of *DEFB1*, *DEFB4*, *Defb1* and *Defb2* could be detected in the appropriate cell lines by 35-cycles of RT-PCR, no significantly increased or reproducible antimicrobial activity could was observed in the β-defensin expressing cell lines compared to control cell lines. This may suggest that recombinant defensin peptides are either non-functional or produced at too low a level to be detected the antibacterial assays. However, the transfected cell lines may express sufficient quantities of β-defensin peptide to possess chemoattractant abilities or for detection by more sensitive antimicrobial assays. Although, if the cell lines are producing such low levels of peptide they may prove to be of little use in further studies. Moreover, a crude western blot analysis did not reveal the presence of additional low molecular weight protein bands not present in the untransfected and empty vector cell lines. If additional protein bands were present, this may have suggested that β-defensin protein was produced but remained non-functional; unfortunately, the absence of correctly controlled antibodies for the β-defensins did not permit more detailed analysis. Furthermore, a Northern blot analysis of 20 μg of also failed to detect the presence of β-defensin mRNA, and this further suggests that β-defensins were not produced at sufficiently high levels to be detectable.

It was not possible to detect expression of *Defb1-4* in the murine C127 cell line, and this permitted easy selection of stably transfected cell lines. In order to help ensure correct cellular processing of the recombinant β-defensins, it

was desirable to stably transfect a cell line that naturally expresses β-defensins. Therefore, attempts were also made to transfect a human bronchial epithelial (HBE) cell line, however, repeated attempts at transfection failed to produce any stable transfected clones. This observation is in agreement with previous findings that the HBE cell line is particularly resistant to stable transfection (Dr Peter Thorpe, University of Edinburgh). It is possible to create stably transfected lines of HBE cells using Mitomycin C-treated feeder cells. Therefore, it may be suitable to generate stable recombinant β-defensin-expressing HBE cell lines using this method as such cells may produce recombinant β-defensins at sufficiently high levels to be of use in further studies.

It is not clear why the transfected C127 cell lines did not produce detectable levels of functional β-defensin peptide. Neither cell lysates nor culture media, even when the potentially inhibitory effects of salt had been removed, demonstrated significantly increased antimicrobial activity compared to the controls. It is possible that any cell line that produced high levels of β-defensin peptide will have been destroyed by cytotoxic levels of the peptide. Indeed α-defensins have been shown to be cytotoxic to mouse cells (Lichtenstein *et al.*, 1986). Of more direct relevance is that DEFB1 has been shown to be cytotoxic to mouse NIH-3T3 cells, although this activity required quite high concentrations and was inhibited by serum (Zucht *et al.*, 1998). It is therefore, perhaps unlikely that the failure to generate cell lines producing high quantities of β-defensins is due to cytotoxicity.

Intriguingly, several studies have suggested that loss of the β-defensin locus may be implicated in the process of tumourigenesis. A study by Abiko *et al.* (1999) demonstrated that some oral squamous cell carcinomas express lower

levels of *DEFB1* and *DEFB4*, however, it was unclear whether transformation induced lower levels of expression of DEFB1 and DEFB4, or if the down-regulation of β-defensin expression caused malignant transformation. Furthermore, a microarray study of renal carcinomas identified *DEFB1* as having a seven-fold lower level of expression than benign tissue (Young *et al.*, 2001). In addition, the chromosomal locus of β-defensins (8p23) is within a region of deletion defined in a loss of heterozygosity study on 96 renal cell carcinomas (Schullerus *et al.*, 1999). These studies suggest that loss of DEFB1, and potentially other β-defensins, may enhance or promote tumour formation. Therefore, it is possible that the growth of the transfected cell lines may be retarded by the β-defensin peptides, and thus low-expressing cell lines may have a growth advantage over those expressing higher levels of the peptide and may grow to dominate the cell cultures.

The potential problems associated with a stably-transfected mammalian cell line expressing β-defensins discussed above may be partially circumvented if the C127 or HBE cells are first stably transfected with the pCMVLacI repressor plasmid (Figure 5.2). Such a system would prevent any expression of the β-defensin until the isopropyl β-D-thiogalactopyranoside (IPTG) stimulant is added to the culture media, and may therefore limit any toxicity or growth-retarding properties. It is interesting to note that a previous and successful attempt to produce β-defensins in mammalian cells used transiently transfected cells and this too would avoid many of the problems that may be associated with stable transfectants. Bals *et al.* (1998a) transiently transfected humans SW13 cells with the Defb1 cDNA, and then demonstrated that transfected cells had significantly increased bactericidal activities compared to empty vector and untransfected controls. However,

the use of transiently transfected cells is less convenient than a stably transfected cell line and thus removes one of the advantages of this system.

Since this study was initiated, it has become clear that it is also possible to produce β-defensins using virally transfected cells. The recombinant DEFB4 used in the study by Bals *et al.* (1999) was produced using a baculovirus system in the Sf9 insect cell line. This generated peptides of two different lengths, one form was 41 amino acids and the other was 38-residues long. However, as this peptide was produced in insect cells and not mammalian cells it is not clear whether the alternative processing represents genuine differences in cellular processing or is an artefact of insect cells. Therefore, to ensure correct cellular processing it is most advantageous to use mammalian cells infected using an adenovirus. This system has been used by Dr Alison Maxwell, working in this laboratory, to express Elafin in cultures of primary murine epithelial cells to investigate its antibacterial activity within airway surface liquid (ASL). It would be of interest to produce β-defensins using this system. This would not only permit the analysis of the antibacterial activity of β-defensins in a more physiologically relevant *in vivo* environment, by incubating bacteria on the surface of transfected cultures, but also to investigate the role of cellular processing on the N-terminal sequence of the mature peptide. The production of large quantities of recombinant β-defensin peptide would allow for its purification and subsequent use in *in vitro* experiments for antibacterial and chemotactic activity such as those presented in Chapters 3 and 4 of this thesis, and a comparison with those obtained with the synthetic peptides. If however, large quantities of β-defensin are required then it may be more suitable to produce the peptides using a bacterial system. Such a system that allows the production of large quantities of easily purified peptide has been described

(Piers *et al.*, 1993). Whilst this study discusses the problems associated with producing and the correct folding of the defensins in bacteria it does not resolve them. However, several recent studies have successful production of β-defensins in bacteria (Harder *et al.*, 2001; Schibli *et al.*, 2002). A His-tag was added to the mature peptide sequence to aid purification and prevent premature activation and this can subsequently be removed by enzymatic digestion. Such a system allows for the production of large quantities of microbicidally active β-defensin peptide of a defined sequence and is likely to prove of great use in future studies, as the method is cost-effective and relatively rapid.

In conclusion, the data presented in this chapter describes an attempt to establish a stably transfected cell line expressing the β-defensins *DEFB1*, *DEFB4*, *Defb1* and *Defb2*. Transfection of the mouse mammary epithelial C127 cell line produced several stable cell lines that, according to RT-PCR analysis, expressed the transfected β-defensin. However, the transfected cell lines did not demonstrate increased antibacterial activity compared to the controls, suggesting that either they did not produce functional β-defensin peptide or did not produce sufficient quantities of β-defensin peptide. It is possible that the transfected cell lines that produced significant quantities of β-defensin peptide were killed by the cytotoxic effects of the β-defensins or their growth was retarded by the β-defensins, which allowed faster growing cells to dominate the culture. It is clearly advantageous to have a recombinant source of β-defensin peptides, and these could be produced either using a bacterial system or by infecting mammalian cells with a viral construct.

Chapter 6: Expression Analysis of Novel Human β-defensins genes

6.1 Introduction

The main human defensin locus is on chromosome 8p22-p23 (Linzmeier *et al.*, 1999; Jia *et al.*, 2001). This region contains both the α- and the β-defensins genes, and it has been suggested that the β-defensins may predate the α-defensins family (Linzmeier *et al.*, 1999). Despite increasing knowledge regarding this locus, to date only four human β-defensins have been described in detail.

All four β-defensin genes show a conserved two exon structure: the first exon encodes most of the propiece, whereas the second encodes part of the propiece and the mature peptide. Substantial differences exist in their primary structures, patterns of expression and spectrums of antimicrobial activity. DEFB1 is constitutively expressed at high levels in the respiratory and urogenital tracts (Bensch *et al.*, 1995); whereas DEFB4 is expressed at high levels in the skin and shows inducible expression in airway epithelial cells in response to microorganisms or tumour necrosis factor-alpha (TNFα) (Harder *et al.*, 1997). Expression of DEFB103 is also inducible, but expression is elevated mostly in response to interferon-gamma (IFNγ). DEFB104 expression, however, appears to be mainly confined to the testis (Garcia *et al.*, 2001b). All β-defensins demonstrate salt-sensitive antimicrobial activity. DEFB1, DEFB4 and DEFB104 show activity against a range of Gram-negative and Gram-positive bacteria reviewed in (Lehrer and Ganz, 2002b). However, whilst DEFB103 shows salt-sensitive antimicrobial activity against Gram-negative bacteria, such as *Escherichia coli*, its activity against the Gram-

positive *S. aureus* appears to be relatively resistant to the inhibitory effects of salt (Harder *et al.*, 2001).

It has been proposed that gene duplication and subsequent functional divergence is one of the most significant processes for the evolution of new gene function (Yokoyama, 2002; Zhang *et al.*, 1998b). However, it remains debatable as to whether the divergence occurs by positive selection (or adaptive evolution), which serves to accelerate fixation of new and advantageous alleles (Goodman *et al.*, 1975), or by random fixation of alleles, which only alter gene function following changes in the environment or genetic background (Dykhuizen and Hartl, 1980). In the latter model the rate of nonsynonymous nucleotide substitutions (*i.e.* those that result in an amino acid change) may be enhanced by relaxation of selective pressure on the redundant duplicated gene, but the rate will not exceed that of synonymous nucleotide changes (*i.e.* those that are silent at the amino acid level). Thus, the two models can be distinguished by comparison of the rates of synonymous and nonsynonymous substitution. By showing that the rate of nonsynonymous substitutions significantly exceeds that of silent synonymous changes, many proteins have been suggested to have undergone positive selection (Yokoyama, 2002). Several examples of gene duplication followed by positive selection have been reported for genes involved in the immune system, where it is argued that the variability generated widens the range and spectra of immune responses. Such examples include the immunoglobulin genes (Ota *et al.*, 2000), and the primate genes for eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), which are thought to have antibacterial and antiviral activities respectively. It has also been proposed that α- and β- defensin genes display such features (Hughes and Yeager, 1997; Hughes, 1999).

The sequencing of the human genome has greatly enhanced the identification of novel genes and the study of gene families. This chapter presents data on the expression analysis of five novel full-length human β-defensin genes identified from the draft human genome sequence (Semple *et al.*, 2002). These candidate genes were identified using bioinformatics techniques by Dr Colin Semple (MRC Human Genetics Unit, Edinburgh).

Previously identified β-defensin sequences from a variety of mammalian species (Table 6.1) were used as BLAST queries against the high throughput genomic (HTG) section of the European molecular laboratory (EMBL) database of human genomic sequence (15th July, 2001 release). All matches were in the region 8p22-p23 and consisted of 53 bacterial artificial chromosome (BAC) sequences; this was deemed to represent the human β-defensin locus and was used in the subsequent analyses. Hidden Markov Models were generated (Eddy, 1998) from a CLUSTALW multiple alignment (Higgins *et al.*, 1996) of known β-defensins, and these models were subsequently used to search against the 53 BAC sequences that represent the human β-defensin locus to identify novel human β-defensin sequences.

All known human β-defensin genes were identified as well as the related epididymis-specific *SPAG11* (initially reported as *EP2*). In addition to these, two novel genes, termed *DEFB105* and *DEFB106* (Figure 6.1 and Table 6.2), were identified; these were incorporated into the Hidden Markov model. Subsequent searches identified a further three β-defensin genes, named *DEFB107*, *DEFB108* and *DEFB109* (Figure 6.1 and Table 6.2).

Species	β-defensin
Human	β-defensin 1, 4, 103 and 104
Mouse (*Mus musculus*)	β-defensin 1-11, 13, 15, 35, Defr1
Rat (*Rattus norvegicus*)	β-defensin 1 and 2
Cow (*Bos taurus*)	β-defensin1-13, TAP, LAP, Enteric β-defensin
Pig (*Sus scrofa*)	β-defensin 1
Sheep (*Ovis aries*)	β-defensin 1 and 2
Goat (*Capra hircus*)	β-defensin 1 and 2
Rhesus monkey (*Macca mulatta*)	β-defensin 1 and 2
Olive baboon (Papio cynocephalus anubis)	β-defensin 1
Chimpanzee (*Pan troglodytes*)	β-defensin 1, 2 and 3

Table 6.1: The Mammalian β-defensins used to construct the Hidden Markov model.

β-defensin		Sequence
DEFB105	cDNA	atggccctgatcaggaagacattttattttctatttgctatgttcttcatt ttggttcaactgccatcaggtgagtttgctgtctgtgagtcgtgcaagctt ggtcggggaaaatgcaggaaggagtgcttggagaatgagaagcccgatgga aattgcaggctgaactttctctgctgcagacagagg
	Pept.	MALIRKTFYFLFAMFFILVQLPSGEFAVCESCKLGRGKCRKECLENEKPDG NCRLNFLCCRQR
DEFB106	cDNA	atgaggactttcctctttctctttgccgtgctcttctttctgaccccaggt aaaatgggcatctttacagggaaggtgatcggagccaagaatgcatttttt gatgagaatgcaacaaacttaaagggacatgcaagaacaattgcgggaaaa atgaaaacttattgctctctgccagaagtctctgaaatgctgtcggaccat c
	Pept.	MRTFLFLFAVLFFLTPGKMGIFTGKVIGAKNAFFDEKCNKLKGTCKNNCGK NEELIALCQKSLKCCRTI
DEFB107	cDNA	atgaaaatatttgtctttattttggctgctctcattcttcttgctcaaatt ttccaagcaattcatagagcactaattagtaagagaatggaaggtcactgt gaagccgaatgtcttacctttgaagtaaagattgggggctgtagagctgaa ttagcaccattttgctgcaaaaacaga
	Pept.	MKIFVFILAALILLAQIFQAIHRALISKRMEGHCEAECLTFEVKIGGCRAE LAPFCCKNR
DEFB108	cDNA	atgaggattgctgtcctcttcttcaccattttcttctttatgagccaagtt ctaccagccaagggcaaattcaaggagatctgtgaacgtccaaatggctcc tgtcgggatttttgcctcgaaacagaaatccatgttgggagatgtttaaat agccgaccctgctgcctgcctctg
	Pept.	MRIAVLFFTIFFFMSQVLPAKGKFKEICERPNGSCRDFCLETEIHVGRCLN SRPCCLPL
DEFB109	cDNA	atgagactccatttgcttctccttattctccttctttttaaattctctta tccccagtaagaggtggtttgggtcctgcggaaggtcattgtctcaatttg tctggtgtttgcagaagagatgtctgcaaagtagtagaagatcaaattggt gcctgccgaagaaggatgaagtgctgtagagcaTGG
	Pept.	MRLHLLLLILLLF*ILLSPVRGGLGPAEGHCLNLSGVCRRDVCKVVEDQIG ACRRRMKCCRAW

Table 6.2: The predicted sequence of novel human β-defensins identified by a BLAST search of the β-defensins locus using hidden Markov models constructed from a CLUSTALW alignment of known β-defensins. Pept.; peptide sequence. *; STOP codon.

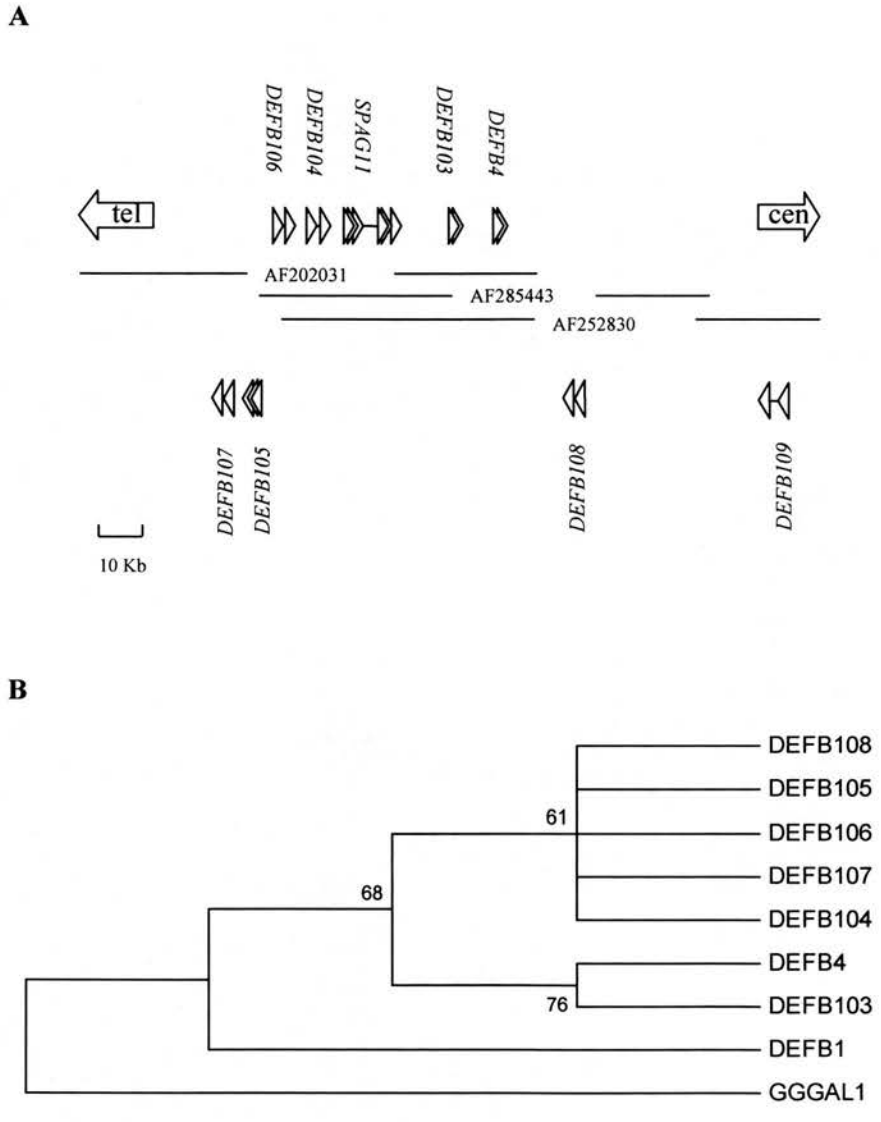

Figure 6.1: (A) The genomic organisation of novel human β-defensin genes *DEFB105*, *DEFB106*, *DEFB107*, *DEFB108* and *DEFB109* on 8p22-p23. Arrow heads show direction of gene orientation, and AF numbers indicate the BAC accession number. **(B)** A phylogenetic tree of functional human β-defensins using the prepropeptide sequences they encode. Figure from Semple *et al.*, (2002). Analysis was rooted using the chicken gallinacin 1 sequence and reproducibility of each branch was assessed 1000 bootstrap replications.

Novel β-defensin genes have been identified at the genomic level but in to order verify whether or not they are genuine it is necessary to investigate if they are expressed in human tissues. In order to do this a panel of human RNAs were obtained and screened by RT-PCR for expression of the novel β-defensins, and in this chapter, the results of this analysis are presented.

Chapter 6.2: Results

Expression analysis of the novel human β-defensin genes was carried out by RT-PCR. A range of human RNA samples from 16 tissues (stomach, kidney, thyroid, lung, skeletal muscle, liver, skin, colon, heart, breast, placenta, uterus, breast, vulva, ovary and testis) was used for this analysis as detailed in the Materials and Methods. Subsequent PCR products were analysed by gel electrophoresis and confirmed by hybridisation to specific internal probes. PCR products were then sequenced to confirm computational predictions of the intron-exon boundaries.

6.2.1 Expression Analysis of novel human β-defensin genes

Expression of all five novel β-defensin genes (*DEFB105, DEFB106, DEFB107, DEFB108, DEFB109*) genes was readily detected in the testis (Figure 6.2). Further analysis revealed expression of *DEFB108* and *DEFB109* in other tissues. *DEFB109* had the most widely distributed expression with low levels of expression detected in the placenta, cervix, thyroid and breast. A low level of *DEFB108* was also detected in the liver.

6.2.2 Sequence Analysis of novel human β-defensins

PCR products from the testis RNA sample were sequenced and the results compared to the sequences identified using the Hidden Markov models to confirm the sequence predicted by computational studies (Figure 6.3). In the case of the sequenced *DEFB105* PCR product, this was 42 bp longer than the cDNA predicted from genomic sequences (Figure 6.3 Ai). The result of this

was to lengthen the predicted peptide by nine amino acid residues (Figure 6.3 Aii). Sequencing of *DEFB106* and *DEFB107* showed that different splice sites than those predicted by the computational analysis were used. This resulted in a 36 base pair (bp) decrease in the predicted coding sequence of *DEFB106,* and a nine bp increase in the predicted sequence of *DEFB107* (Figure 6.3 Bi and Ci). Consequently, the DEFB106 peptide contains 12 fewer residues and the DEFB107 peptide is three amino acids longer than initially predicted (Figure 6.3 Bii and Cii). Sequenced DEFB108 contained three nucleotide substitutions, two of which (T→C at position 111 and C→T at 120) were silent but the substitution of an adenine for a guanine at position 61, inserts an arginine (R) in the place of the predicted (K) (Figure 6.3 D). Strikingly, sequencing of *DEFB109* generated two different transcripts. One form was identical to the predicted *DEFB109* cDNA, however the alternate *DEFB109* sequence had a base substitution at position 41, which in the peptide replaced stop codon with a serine residue. The transcript also had three additional amino acid substitutions as a result of further nucleotide changes (Figure 6.3 E). All sequences were confirmed by the existence of at least two independently sequenced clones.

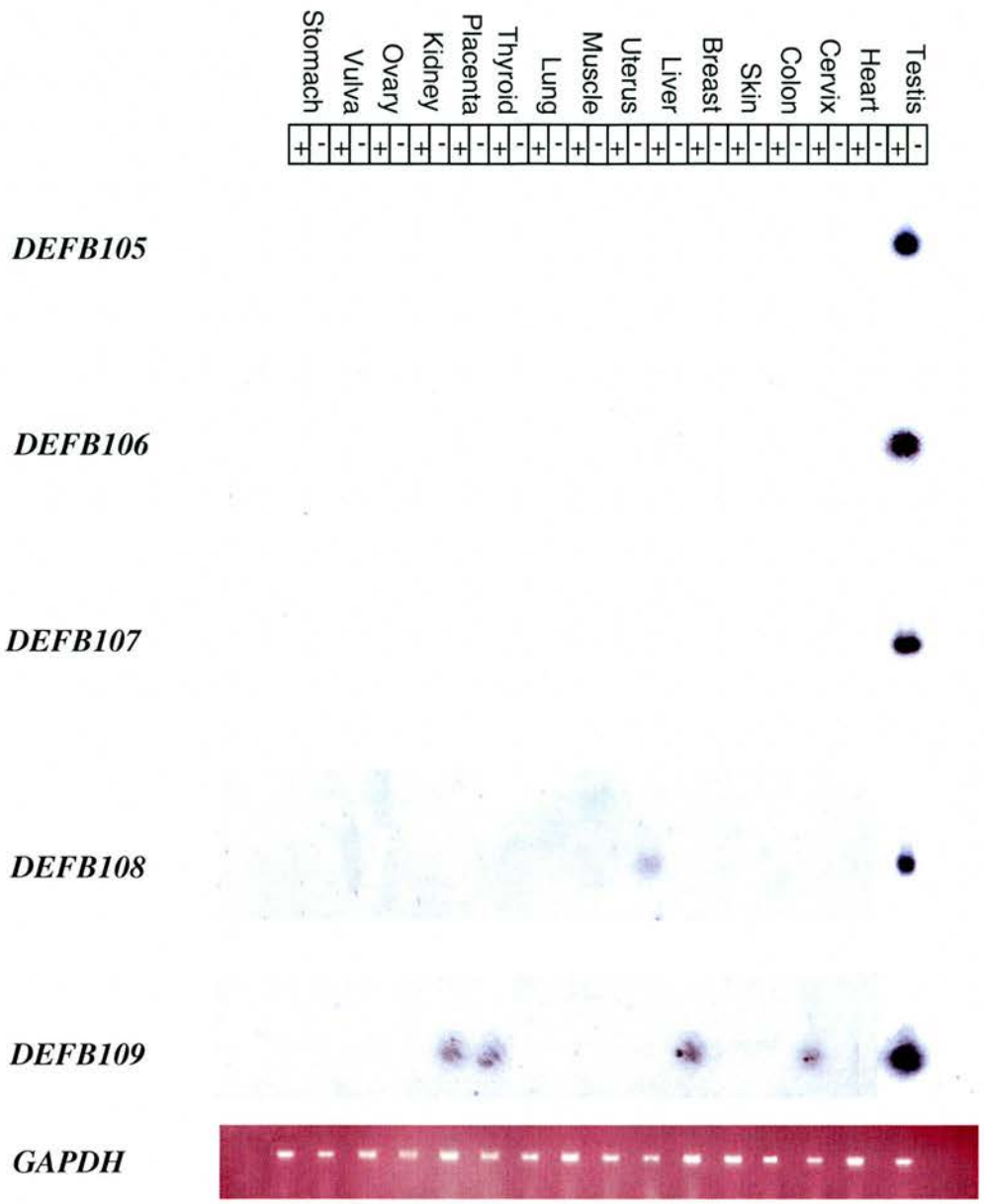

Figure 6.2: RT-PCR Analysis of novel human β-defensin gene expression

RT-PCR was carried out on a panel of human RNAs. Reactions were set with either with (+) or without (-) reverse transcriptase. GAPDH was carried out as a PCR control. RT-PCR was repeated three times to ensure reproducibility of the result (n=3).

A) *DEFB105*

```
i.) Percent Similarity: 98.734
                .         .         .         .         .
      28 tttctatttgctatgttcttcattttggttcaactg..............  63
         ||||||||||||||||||||||||||||||||||
         TTTCTATTTGCTATGTTCTTCATTTTGGTTCAACTGCCATCAGGGTGCCA
                .         .         .         .         .
      64 ............................ccatcaggtgagtttgctgtct  85
                                     |||||||||||||||||||||
         GGCAGGACTTGATTTTTCCCAACCATTTCCATCAGGTGAGTTTGCTGTCT
                .         .         .         .         .
      86 gtgagtcgtgcaagcttggtcggggaaaatgcaggaaggagtgcttggag 135
         |||||||||||||||||||||||||||||||||||||||||||||||||
         GTGAGTCGTGCAAGCTTGGTCGGGGAAAATGCAGGAAGGAGTGCTTGGAG
                .         .         .         .         .
     136 aatgagaagcccgatggaaattgcaggctgaactttctctgctgcagaca 185
         ||||||||||||||||||||||||||||||||||||||||||||  ||
         AATGAGAAGCCCGATGGAAATTGCAGGCTGAACTTTCTCTGCTGCAATCA

ii.) Percent Similarity: 100.000
                .         .         .         .         .
      10 flfamffilvql..............psgefavcescklgrgkcrkecle  45
         ||||||||||||              ||||||||||||||||||||||||
         FLFAMFFILVQLPSGCQAGLDFSQPFPSGEFAVCESCKLGRGKCRKECLE
                .
      46 NEKPDGNCRL  55
         ||||||||||
         NEKPDGNCRL
```

B) *DEFB106*

```
i.) Percent Similarity: 100.000
                .         .         .         .         .
      27 cgtgctcttctttctgaccccaggtaaaatgggcatctttacagggaagg  76
         ||||||||||||||||||||
         CGTGCTCTTCTTTCTGACCCC..........................
                .         .         .         .         .
      77 tgatcggagccaagaatgcatttttgatgagaaatgcaacaaacttaaa 126
                 ||||||||||||||||||||||||||||||||||||||||||
         .......AGCCAAGAATGCATTTTTTGATGAGAAATGCAACAAACTTAAA
                .         .         .
     127 gggacatgcaagaacaattgcgggaaaaatgaagaac 163
         |||||||||||||||||||||||||||||||||||
         GGGACATGCAAGAACAATTGCGGGAAAAATGAAGAAC

ii.) Percent Similarity: 100.000
                .         .         .         .
      10 vlffltpgkmgiftgkvigaknaffdekcnklkgtcknncgknee  54
         |||||||            |||||||||||||||||||||||||
         VLFFLTP............AKNAFFDEKCNKLKGTCKNNCGKNEE
```

Figure 6.3: Sequencing of *DEFB105* and *DEFB106* novel human β-defensin genes and comparison to predicted genes

Sequences in lower case letters indicate the predicted sequence generated by computational analysis; sequence in capital letters represents the sequenced RT-PCR products. The PCR products generated from the testis RNA sample were cloned and sequenced as detailed in materials and methods. Ai and Bi represent the DNA sequence of the genes compared to the predicted sequences generated by computational studies. Comparisons were performed using the Bestfit function in the GCG program at HGMP (www.hgmp.mrc.ac.uk) with default settings. Aii and Bii show the peptide sequence of the genes. These are compared to the predicted amino acid sequences based on the novel genes.

A) *DEFB107*

```
i.) Percent Similarity: 98.137
                .         .         .         .         .
      14 tctttattttggctgctctcattcttcttgctcaaattttcca..... 56
         ||  | ||||||||||||||||||||||||||||||||||||
         TCGATTTTTTGGCTGCTCTCATTCTTCTTGCTCAAATTTTCCAAGCCAGG
                .         .         .         .         .
      57 ..agcaattcatagagcactaattagtaagagaatggaaggtcactgtga 104
           ||||||||||||||||||||||||||||||||||||||||||||||||
         ACAGCAATTCATAGAGCACTAATTAGTAAGAGAATGGAAGGTCACTGTGA
                .         .         .         .         .
     105 agccgaatgtcttacctttgaagtaaagattgggggctgtagagctgaat 154
         ||||||||||||||||||||||||||||||||||||||||||||||||||
         AGCCGAATGTCTTACCTTTGAAGTAAAGATTGGGGGCTGTAGAGCTGAAT
                .         .
     155 tagcaccattttgctgcaaa 174
         ||||||||||||||||||||
         TAGCACCATTTTGCTGCAAA

ii.) Percent Similarity: 98.039
                .         .         .         .         .
       8 laalillaqifq...aihraliskrmeghceaecltfevkiggcraelap 54
         ||||||||||||   |||||||||||||||||||||||||| |||||||
         LAALILLAQIFQARTAIHRALISKRMEGHCEAECLTFEVKIGVCRAELAP

      55 fcck 58
         ||||
         FCCK
```

B) *DEFB108*

```
 i.) Percent Similarity: 98.026
                .         .         .         .         .
       8 ttgctgtcctcttcttcaccattttcttctttatgagccaagttctacca 57
         ||||||||||||||||||||||||||||||||||||||||||||||||||
         TTGCTGTCCTCTTCTTCACCATTTTCTTCTTTATGAGCCAAGTTCTACCA 57
                .         .         .         .         .
      58 gccaagggcaaattcaaggagatctgtgaacgtccaaatggctcctgtcg 107
         |||| ||||||||||||||||||||||||||||||||||||||||||||
         GCCAGGGGCAAATTCAAGGAGATCTGTGAACGTCCAAATGGCTCCTGTCG
                .         .         .         .         .
     108 ggatttttgcctcgaaacagaaatccatgttgggagatgtttaaatagcc 157
         ||| |||||||| ||||||||||||||||||||||||||||||||||||
         GGACTTTTGCCTTGAAACAGAAATCCATGTTGGGAGATGTTTAAATAGCC

     158 ga 159
         ||
         GA

ii.) Percent Similarity: 100.000
                .         .         .         .         .
       4 avlfftifffmsqvlpakgkfkeicerpngscrdfcleteihvgrclnsr 53
         |||||||||||||||||:||||||||||||||||||||||||||||||||
         AVLFFTIFFFMSQVLPARGKFKEICERPNGSCRDFCLETEIHVGRCLNSR
```

Figure 6.4: Sequencing of *DEFB107* and *DEFB108* novel human β-defensin genes and comparison to predicted genes

Sequences in lower case letters indicate the predicted sequence generated by computational analysis; sequence in capital letters represents the sequenced RT-PCR products. The PCR products generated from the testis RNA sample were cloned and sequenced as detailed in materials and methods. Ai and Bi represent the DNA sequence of the genes compared to the predicted sequences generated by computational studies. Comparisons were performed using the Bestfit function in the GCG program at HGMP (www.hgmp.mrc.ac.uk) with default settings. Aii and Bii show the peptide sequence of the genes. These are compared to the predicted amino acid sequences based on the novel genes.

A) *DEFB109*

```
i.) Percent Similarity: 100
                .         .         .         .         .
       2 tgagactccatttgcttctccttattctccttctttttaaattctctta 51
         ||||||||||||||||||||||||||||||||||||||||||||||||||
         TGAGACTCCATTTGCTTCTCCTTATTCTCCTTCTTTTTTAAATTCTCTTA
                .         .         .         .         .
      52 tccccagtaagaggtggtttgggtcctgcggaaggtcattgtctcaattt 101
         ||||||||||||||||||||||||||||||||||||||||||||||||||
         TCCCCAGTAAGAGGTGGTTTGGGTCCTGCGGAAGGTCATTGTCTCAATTT
                .         .         .         .         .
     102 gtctggtgtttgcagaagagatgtctgcaaagtagtagaagatcaaattg 151
         ||||||||||||||||||||||||||||||||||||||||||||||||||
         GTCTGGTGTTTGCAGAAGAGATGTCTGCAAAGTAGTAGAAGATCAAATTG

     152 gtgcctgc 159
         ||||||||
         GTGCCTGC

ii.) Percent Similarity: 100
                .         .         .         .         .
       2 rlhlllllllf*illspvrgglgpaeghclnlsgvcrrdvckvvedqig 51
         |||||||||||| ||||||||||||||||||||||||||||||||||||
         RLHLLLLILLLF*ILLSPVRGGLGPAEGHCLNLSGVCRRDVCKVVEDQIG

      52 ac 53
         ||
         AC
```

B) **Alternate '*DEFB109*' sequence**

```
i.) Percent Similarity: 97.468
                .         .         .         .         .
       2 tgagactccatttgcttctccttattctccttctttttaaattctctta 51
         |||||||||||||||||||||||||||||||||||| ||||||||||
         TGAGACTCCATTTGCTTCTCCTTATTCTCCTTCTTTTTTCAATTCTCTTA
                .         .         .         .         .
      52 tccccagtaagaggtggtttgggtcctgcggaaggtcattgtctcaattt 101
         |||||||||||||||| ||||||||||||||| |||||||||| |||
         TCCCCAGTAAGAGGTGCTTTGGGTCCTGCGGAAGTTCATTGTCTCAGTTT
                .         .         .         .         .
     102 gtctggtgtttgcagaagagatgtctgcaaagtagtagaagatcaaattg 151
         ||||||||||||||||||||||||||||||||||||||||||||||||||
         GTCTGGTGTTTGCAGAAGAGATGTCTGCAAAGTAGTAGAAGATCAAATTG

     152 gtgcctgc 159
         ||||||||
         GTGCCTGC

ii.) Percent Similarity: 92.308
                .         .         .         .         .
       2 rlhlllllllf*illspvrgglgpaeghclnlsgvcrrdvckvvedqigac53
         |||||||||||| |||||||| ||||| |||.||||||||||||||||||
         RLHLLLLILLLFSILLSPVRGALGPAEVHCLSLSGVCRRDVCKVVEDQIGAC
```

Figure 6.5: Sequencing of *DEFB109* and alternate *DEFB109* novel human β-defensin genes and comparison to predicted genes

Sequences in lower case letters indicate the predicted sequence generated by computational analysis; sequence in capital letters represents the sequenced RT-PCR products. Ai and Bi represent the DNA sequence of the genes compared to the predicted sequences generated by computational studies. Sequences and alignments were generated as previously described (See Figures 6.3 and 6.4). * indicates a STOP codon .

Chapter 6.3: Discussion

This chapter presents data on the expression pattern of five novel human β-defensin genes, *DEFB105 – 109*. The identification of the genes and subsequent computational and evolutionary analysis was conducted by Dr Colin Semple, MRC Human Genetics Unit.

All five of the predicted genes were found to be expressed in the testis and *DEFB108* and *DEFB109* were also expressed in other tissues but at much lower levels. It is not clear whether these levels of expression are functional and further work would be required to clarify this issue. Moreover, it is not clear if expression of any of these genes can be induced by inflammatory stimuli or cytokines, and therefore further analysis is required to investigate if expression of these novel genes shows inducible expression in other tissues.

It is interesting to note that expression of all of these novel genes was readily detected in the testis as this appears to be a common site for expressions of β-defensins and β-defensin like molecules. In humans, *DEFB103* and *DEFB104* show very high levels of expression in the testis (Garcia *et al.*, 2001a; Garcia *et al.*, 2001b), and *DEFB1* is also expressed in the testis, but at much lower levels (Zhao *et al.*, 1996). Expression of *DEFB4* in the testis has not been reported. Furthermore, in the mouse, *Defb3* (Bals *et al.*, 1999) and *Defensin-related 1* (*Defr1*) (Morrison *et al.*, 2002a) also show high levels of expression in the testis.

Expression of several β-defensin-like genes has also been reported in the testis. In humans, the androgen-dependent epididymis-specific secretory

protein *SPAG11* (*EP2*) is expressed in the testis and is suggested to have a functional role in sperm maturation or storage (Frohlich *et al.*, 2001; Lehrer and Ganz, 2002b). The *SPAG11* gene encodes at least nine mRNA variants; it has two promoters, eight exons and seven introns. Exons 3 and 6 contain sequences homologous to β-defensins and it has been suggested that *SPAG11* evolved from two tandem β-defensin genes reviewed in (Lehrer and Ganz, 2002b). It is interesting to note that many of the testis-specific β-defensin genes described in this chapter (*i.e. DEFB105-107*) are clustered around the *SPAG11* gene in the human genome, and may represent an epididymis-specific β-defensin region (Yamaguchi *et al.*, 2002). Furthermore, a β-defensin-like gene, called *Binb1*, has also been identified in the rat epididymis (Li *et al.*, 2001). *Binb1* has been found to be expressed exclusively in the caput region of the epididymis and interestingly, this area is responsible for sperm maturation and storage. The gene is maximally expressed when the rats are sexually mature and is induced by inflammatory stimuli. It is proposed to function as an antimicrobial peptide. A β-defensin-like gene has also identified in the mouse epididymis. The testis-specific β-defensin-like gene (*Tdl*), which encodes an 83 amino acid peptide and shows the conserved 6-cysteine motif of β-defensins, was found to be specifically expressed in the Sertoli cell-lineage in the seminiferous cords of the male gonads when sexual differentiation is induced in the developing gonads (Yamamoto and Matsui, 2002). It would be of interest to investigate whether β-defensins expressed in the testis play a structural role or exhibit a function other than as an aspect of the immune system.

The PCR products of these novel genes were also sequenced and some showed striking differences compared to the predicted genes. The sequenced PCR products for *DEFB105* showed a 42 base pair (bp) increase in

the predicted length of the peptide, most notably this additional sequence produces an additional cysteine, giving the predicted DEFB105 mature peptide a total of seven as opposed to the normal conserved six (Figure 6.5). Moreover, this extra sequence formed an additional exon, giving the *DEFB105* gene three exons compared to the two exons that comprise all other β-defensins characterised to date. Exons 1 to 3 of the *DEFB105* gene are 70, 42 and 119 bp long respectively. The first intron is only 334 bp long and the second 1200 bp. In is interesting to note that the genes for the α-defensin human neutrophils peptides, *HNP1-4*, are composed of three exons (Linzmeier *et al.*, 1993).

When the PCR products for the gene *DEFB109* were sequenced, two different sequences were identified. One *DEFB109* transcript is identical to the predicted *DEFB109* sequence. This sequence shows a stop codon in the peptide at position 14, this stop codon was present in three independently sequenced and overlapping BAC sequences (accession numbers AC068974, AC087203 and AF252830), and therefore *DEFB109* is predicted to be a pseudogene. However, the alternate transcript amplified by the *DEFB109* PCR primers has a serine residue substituted for the stop codon and the presence of an additional three amino acid substitutions compared to the predicted sequence for *DEFB109*. Interestingly, a BLAST search analysis shows that the alternate transcript of *DEFB109* sequence matches exactly to a gene on the BAC (accession number AC092450), which is localised to chromosome 12 and therefore termed *DEFB-like*^{Chr12} (Figure 6.6). This gene is also composed of two exons and the exonic boundaries match those predicted for *DEFB109*. Chromosome 12 does not contain any known β-defensin loci, and may represent a new locus of β-defensins, however this

region remains poorly characterised in terms of mapping. Repeat sequence analysis of the PCR products from the testis RNA revealed both *DEFB109* and *DEFB-like*^{Chr12} clones. Therefore, the expression of DEFB109 detected in the placenta, cervix, thyroid and breast may be exclusively *DEFB109* or *DEFB*-like^{Chr12} or, as in the testis, a mixture of the two different transcripts. Analysis of the PCR products form these tissues with oligonucleotide probes designed exclusively to *DEFB109* or *DEFB-like*^{Chr12} transcripts should distinguish the expression profile of these genes.

The sequence derived from the PCR products of *DEFB106* and *DEFB107* also deviated from the predicted sequences. The product for *DEFB106* contained 36 fewer nucleotides in the first exon compared to the predicted sequence due to a different donor site than was predicted (Figure 6.3 Bi), resulting in 12 fewer amino acid residues than in the predicted peptide. In contrast, DEFB107 contained three extra amino acids due to nine additional base pairs in the second exon, due to a different acceptor site than the one predicted by computational analysis. Furthermore, the sequence generated from the *DEFB107* PCR product suggests that the DEFB107 peptide lacks the first canonical cysteine. This is of particular interest given that it has been shown that the loss of the first cysteine in the murine Defensin-related-1 peptide (Defr1) does not result in the loss of antibacterial function. Indeed, Defr1 appears to possess particularly potent antimicrobial activity against *P. aeruginosa* and it also shows activity against the normally resistant *B. cenocepacia* J2315 (Morrison *et al.*, 2002a). Sequencing of *DEFB108* also showed differences compared to the predicted structures, however, only one of which resulted in an amino acid change with arginine replacing the predicted lysine. Interestingly, such a change would retain the overall charge of the peptide as both lysine and arginine are positively charged

amino acids, and therefore this change may result in only minor alterations in the antibacterial activity of the peptide.

```
          tgagactccatgtgcttctccttattctccttctttttcaattctctta
          |||||||||||  ||||||||||||||||||||||||||||||||||||
670064    TGAGACTCCATTTGCTTCTCCTTATTCTCCTTCTTTTTTCAATTCTCTTA

           tccccaggtaag
           ||||||||||||
670013     TCCCCAG-TAAG

            cagtaagaggtgctttgggtcctgcggaagttcattgtctcagtttgtctg
            |||||||||||||||||||||||||||||||||||||||||||||||||||
663164      CAGTAAGAGGTGCTTTGGGTCCTGCGGAAGTTCATTGTCTCAGTTTGTCTG

            gtgtttgcagaagagatgtctgcaaagtagtagaagatcaaattggtgcctgc
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
663113      GTGTTTGCAGAAGAGATGTCTGCAAAGTAGTAGAAGATCAAATTGGTGCCTGC

            gc
            ||
663062      GC
```

Figure 6.6: BLAST search of *DEFB-like*^{chr12} sequence identifies a gene contained on a BAC located to chromosome 12. The sequence in lowercase lettering represents the input sequence of *DEFB109*^{Ch12}, the sequence in uppercase lettering represents the sequence on BAC AC068974. The search was conducted using BLAST function at the NCBI website.

While the work presented in this chapter was carried out, Schutte *et al.*, published a wide spread analysis of β-defensin genes in the human and mouse genomes (Schutte *et al.*, 2002). This study was able to identify 28 novel human and 43 novel mouse final exon fragments. Whilst this data identified the second exons for the genes reported in this chapter, however, first exon sequences were not identified, and neither were the boundaries of the second exons elucidated. Moreover, the data presented by Schutte *et al.* was not verified by experimental analysis of the patterns of expression. Most interestingly, however, the novel β-defensin genes identified by Schutte *et al.* were found at five different chromosomal locations and these loci were in syntenic regions on human and mouse chromosomes. A search of the human

defensin locus at 8p22-23 revealed the five genes presented in this chapter, Schutte *et al.* named these *DEFB5-9* (in this chapter the nomenclature established by the Human Genome Nomenclature Committee has been adopted and the genes referred to as *DEFB105-109).* Wider searches, incorporating the novel genes, of the draft human genome identified a further five β-defensins genes (named *DEFB101-4*) that located to the region 6p12, and subsequent searches revealed another 15 β-defensin genes (*DEFB16 – DEFB29*) at two more locations: 20q11.1 and 20p13. Finally, subsequent analysis of the entire genome with a hidden Markov model incorporating all previously identified genes, revealed the existence of a further 40 6-cysteine genes. However, in only two cases was the spacing of the cysteines and the cationic charge consistent with β-defensin genes, these were named *DEFB30* and *DEFB31* and these genes were also found to locate to 8p22-23, although this region was not contiguous with the original cluster and thus may represent a fifth β-defensins cluster (Schutte *et al.*, 2002). The same procedure was applied to searches conducted of the mouse genome, which revealed the existence of 43 novel β-defensins genes on chromosomes 1, 8, 14 and two clusters on chromosome 2. Interestingly, these regions are syntenic with those previously identified in humans (Schutte *et al.*, 2002), suggesting that these clusters have a common origin.

A very recent report also identified *DEFB105* and *DEFB106* (reported as *hBD-5* and *hBD-6*), confirmed their expression as testis-specific, and the three-exon structure of *DEFB105* was also reported by this study (Yamaguchi *et al.*, 2002). Moreover, the authors also identified the murine homologues of *DEFB105* and *DEFB106* as *mBD12* (*Defb12*) and *mBD11* (*Defb11*) respectively, which were isolated as partial sequences from the Schutte *et al.* study (2002)

and interestingly the *Defb12* gene (homologue of *DEFB105*) was also found to be composed of three exons.

As was discussed earlier the five novel β-defensin genes presented here locate to the region 8p22-23 (Figure 6.1). A phylogenetic tree of human β-defensins (Figure 6.5) reflects the spatial arrangement of these genes (Figure 6.1). The genes *DEFB104-7*, which form one a separate cluster from *DEFB103* and *DEFB4* (Figures 6.1B, 6.7 and 6.8) are clustered in together a group within region 8p22-23, whereas the other cluster formed by *DEFB4* and *DEFB103* are located in a region centromeric to the larger cluster. This may suggest that this cluster was generated by a series of local duplications and subsequent divergence. It also appears that *DEFB105-109* are more closely related to *DEFB104* than to *DEFB4* or *DEFB103*. Moreover, the spacing of cysteines in *DEFB104* are slightly different to that in DEFB1 and DEFB4, in that there is one fewer amino acid residue between the fourth an fifth cysteines. Three of the novel genes, *DEFB105*, *DEFB106* and *DEFB108*, show this same spacing of the cysteines, although DEFB105 contains an additional cysteine (residue 43) and has an unusually long propiece due to the additional exon (Figure 6.7).

Interestingly, six genes that display high levels of similarity (85-98%) to three of the novel β-defensins presented in this chapter (*DEFB105*, *DEFB106* and *DEFB107*) were recently identified in two olive baboon (*Papio cynocephalus anubis)* draft genome sequences (Figure 6.7). These genes also displayed high levels of similarity to the previously identified β-defensin genes *DEFB4*, *DEFB103* and *DEFB104*. It is likely that these genes originate from the

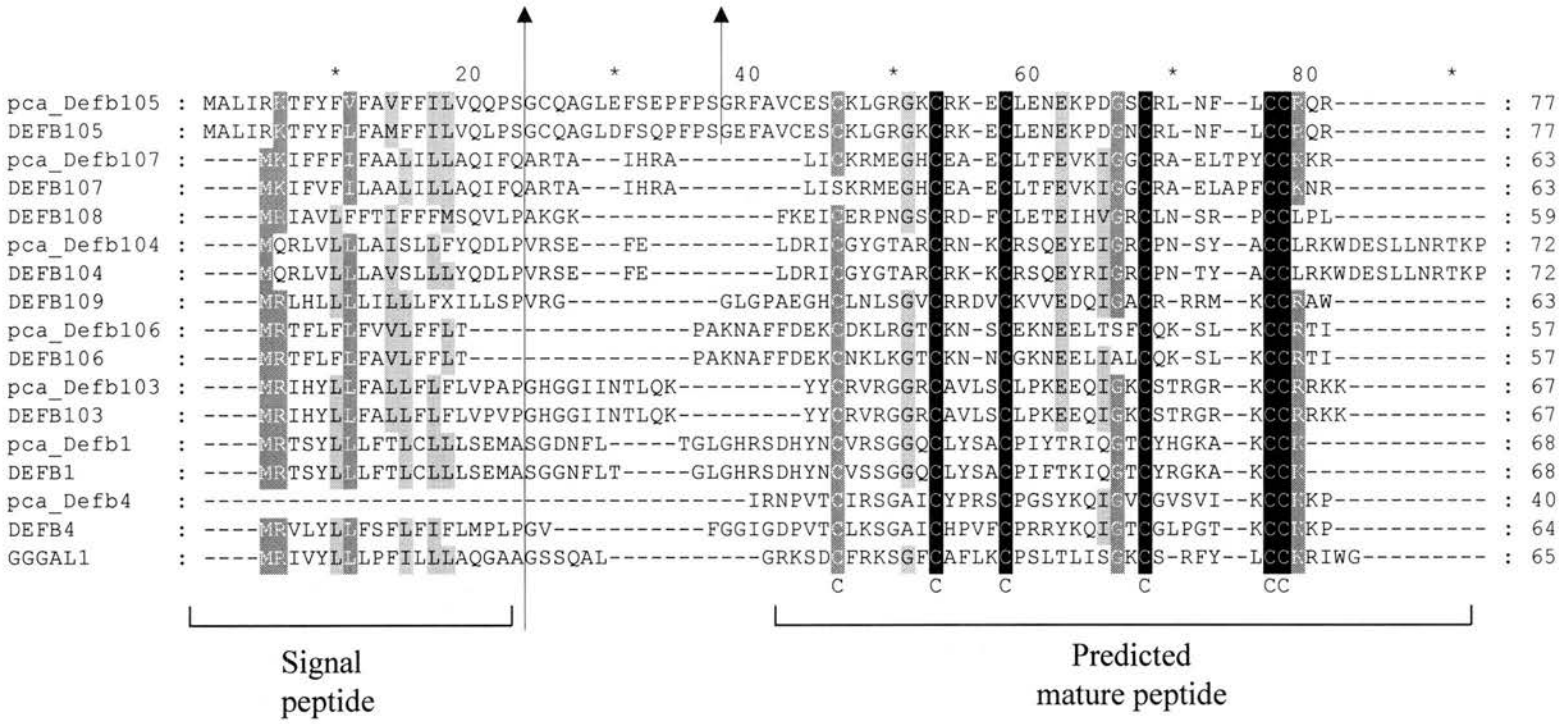

Figure 6.7: Alignment of human and baboon β-defensin protein sequences. The alignment shows the same sequences with the estimated locations of the signal peptide and the predicted mature peptide regions, the intervening region is the propiece. Arrows indicate the approximate position of introns, with the short arrow indicating the second intron found only in *DEFB105*. The shading represents the degree of conservation at each position in the alignment, taking into account similar physiochemical properties of residues. The six canonical cysteines are indicated under the appropriate alignment positions. pca = olive baboon (*Papio cyanocephalus anubis*).

baboon locus syntenic to the human region 8p22-p23, although more detailed mapping is required to confirm this.

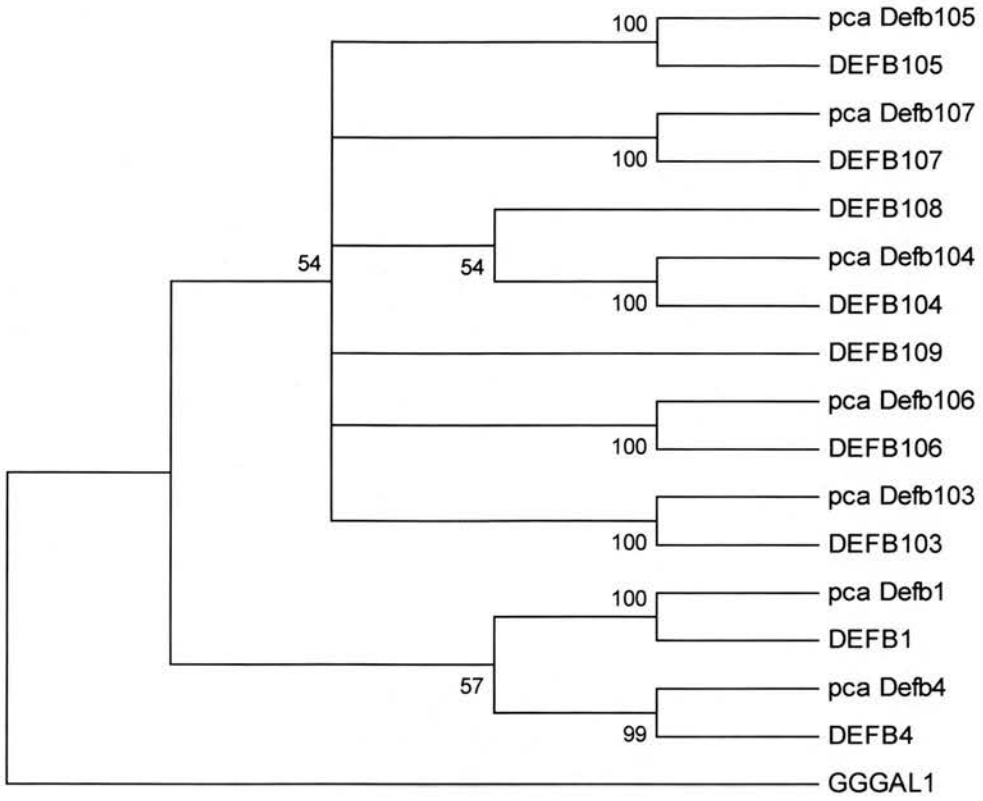

Figure 6.8: Phylogenetic tree of human and baboon β-defensin protein sequences. The tree was rooted with chicken gallinacin 1 (*GGGAL1*) and the reliability of each branch was assessed using 1000 bootstrap replications. pca = olive baboon (*Papio cyanocephalus anubis*). Figure taken form Semple *et al.* (2002)

As was discussed earlier it has been suggested that the evolution of the defensins has occurred by duplication with subsequent divergence driven by positive selection. This pattern of evolution has also been observed for rat, murine and guinea pig α-defensins and also antimicrobial peptides of ranid and hylid frog species (Hughes and Yeager, 1997; Duda, *et al.*, 2002). An analysis of the rate of synonymous (d_S) and nonsynonymous substitutions (d_N) in the first and second exons of human and baboon β-defensins was

conducted, according to the method of Nei and Gojobori (1986), and two different patterns were revealed (Semple *et al.*, 2002). In the first exon, which encodes the signal sequence of the peptide, the rates of synonymous substitution were greater than the rates of nonsynonymous substitutions. This suggests that they are evolving under neutral or possibly very weak purifying, selection. In the second exon, however, which encodes the mature peptide a quite different trend was observed; the rate of nonsynonymous substitutions exceeded those of synonymous (Table 6.3). Moreover, this pattern was observed for the human β-defensins and the orthologous baboon genes. Significant excesses of d_N over d_S are seen between *DEFB1* and *DEFB104* and also between *DEF103* and *DEFB107* these comparisons were statistically significant according to the Z-test, and with the exception of *DEFB103* versus *DEFB107* also according to the more rigorous Fischer's exact test (Table 6.3). However, it is difficult to achieve statistical significance owing to the short length of these peptides.

This pattern suggests that the duplication events, which gave rise to the different β-defensins, were followed by rapid diversification driven for positive selection for amino acid substitutions. Furthermore, a comparison of the ratios of radical and conservative amino acid changes (P_R/P_C) in the second exons of genes showing evidence of positive selection were also analysed (Table 6.2). Averages for the P_R/P_C assessed across 47 genes were reported as 0.81 and 0.49 for charge and the Miyata-Yasunaga classification (polarity and volume) respectively (Zhang, 2000). The rates calculated for the β-defensin genes suggesting positive selection greatly exceeded the rates reported by Zhang (2000). Moreover, there appears to have been a predisposition for amino acid changes that alter charge rather than polarity and volume (Table 6.3). This suggests that, where there is evidence of

positive selection, most nonsynonymous changes have tended to result in an alteration of amino acid charge and retention of polarity and volume, such substitutions were also seen in the α-defensins and frog antimicrobial peptides (Hughes and Yeager, 1997; Duda, *et al.*, 2002). The evolution of α-defensins is marked by a pattern of compensation by the propiece for the mature peptide. It has been suggested that the anionic propiece serves to balance the cationicity of the mature peptide and thus maintains a neutral charge in the propeptide, which serves to prevent premature activation of the defensin whilst it is stored in the granules of neutrophils (Valore *et al.*, 1996).

	DEFB1 vs *DEFB104*		*DEFB103* vs *DEFB107*	
	Human	**Olive baboon**	**Human**	**Olive baboon**
S[a]	17.917 ± 1.180	17.167 ± 1.169	16.917 ± 1.165	16.833 ± 1.124
N	48.083 ± 1.144	48.833 ± 1.181	49.083 ± 1.156	49.167 ± 1.124
s	6.25 ± 1.954	6.25 ± 1.954	4.5 ± 1.796	4 ± 1.767
n	35.75 ± 3.482	34.75 ± 3.559	27.5 ± 3.382	27 ± 3.373
d_S	0.349 ± 0.103	0.364 ± 0.106	0.266 ± 0.104	0.238 ± 0.105
d_N	0.744 ± 0.062	0.712 ± 0.063	0.560 ± 0.064	0.549 ± 0.067
Z-test	0.001	0.005	0.020	0.008
Fisher's	0.012	0.019	0.060	0.023
Charge[b]				
p_C	0.498 ± 0.088	0.535 ± 0.102	0.447 ± 0.094	0.409 ± 0.094
p_R	0.784 ± 0.077	0.830 ± 0.086	0.571 ± 0.087	0.575 ± 0.085
p_R / p_C	1.57*	1.55*	1.28	1.41
M-Y[c]				
p_C	0.577 ± 0.116	0.708 ± 0.108	0.598 ± 0.124	0.581 ± 0.124
p_R	0.618 ± 0.092	0.634 ± 0.100	0.467 ± 0.080	0.446 ± 0.082
p_R / p_C	1.07	0.90	0.78	0.77

Table 6.3: Estimated distances and other parameters for comparisons between the second exons of human and baboon genes demonstrating positive selection. [a] Estimates (±SE) of the number of synonymous sites (S), number of nonsynonymous sites, numbers of synonymous substitutions (s), numbers of nonsynonymous substitutions (n), the number of synonymous substitutions per synonymous site (d_S) and the number of nonsynonymous substitutions per nonsynonymous site (d_N). The result of a two-tailed Z-test ($d_S - d_N=0$), and of a Fisher's exact test are also included. [b] Rates of radical (p_R) and conservative (p_C) changes in amino acid properties, with the ratio of radical to conservative changes (p_R / p_C) for residues categorised in terms of their charges. Asterices indicate where p_R is significantly greater than p_C. [c] Rates of radical (p_R) and conservative (p_C) changes in amino acid properties, with the ratio of radical to conservative changes (p_R / p_C) for residues categorised in terms of the Miyata-Yasunaga classification (M-Y; a combination of polarity and volume). Figure taken form Semple *et al.* (2002)

Moreover, it has been proposed that the evolution of the α-defensins has occurred in a coordinated fashion to preserve the charge balance between the propiece and mature peptide; with changes in the net positive charge of the mature peptide balanced by corresponding changes in the propiece (Hughes and Yeager, 1997). However, a similar pattern of coordinated evolution is not seen in the β-defensins, this is most probably because the β-defensins are not stored in granules prior to release and therefore there is no requirement for the propiece to neutralise the charge of the mature peptide to inhibit its activity. Similar patterns of adaptive evolution is often observed in genes associated with the immune system such as lysozyme (Messier and Stewart, 1997), the major histocompatibility complex (Hughes and Nei, 1988) and also in amphibian antimicrobial peptides (Duda *et al.*, 2002). However, some of the highest rates of adaptive evolution are observed in genes associated with testis function and fertility (Johnson *et al.*, 2001).

In summary, this chapter has presented data on the expression pattern of five novel β-defensin genes. Expression of all five genes was readily detected in the testis, and *DEFB108* and *DEFB109* were also expressed at lower levels in other tissues. Sequencing of these genes revealed striking differences compared to the computationally predicted gene structure. The results of sequencing revealed that the *DEFB105* gene is composed of three exons, whereas all previously characterised β-defensin genes are comprised of two exons. Sequencing analysis also showed that RT-PCR analysis of *DEFB109* actually amplified two different transcripts; one transcript matched the predicted *DEB109*, this transcript contains a stop codon and may therefore represent a pseudogene. The *DEFB-likeChr12* transcript contained a serine residue in place of the predicted stop codon and three further amino acid changes compared to the predicted *DEFB109*[12] sequence; a BLAST search

suggested that this sequence matched exactly to an unknown gene on chromosome 12. Clearly, further analysis is required to investigate to what extent the detected expression of *DEFB109* is due to the predicted *DEFB109* or to *DEFB109*^{Chr12}. One of the novel β-defensin genes, DEFB107, lacks the first canonical cysteine and this is of particular interest in the light of the isolation of murine Defr1, which lacks the first cysteine and the observation that it displays highly potent anti-pseudomonal activity (Morrison *et al.*, 2002a). Moreover, the predicted DEFB105 peptide contains an additional cysteine and investigation of the antibacterial activity and structure of these novel peptides would be of great interest.

7. Conclusions and Future Studies

Antimicrobial peptides comprise an important aspect of innate immune system of mammals. The β-defensins are a subfamily of antimicrobial peptides produced by epithelial tissues. Data presented in this thesis further supports the notion that β-defensins are a large family of salt-sensitive antimicrobial peptides, with many members as yet uncharacterised. It has also been shown that murine β-defensins also have chemoattractant ability *in vitro* for cells linked to the adaptive immune system.

This thesis has presented studies investigating the antimicrobial activity of synthetic murine β-defensin Defb2, the human β-defensin DEFB4 and the novel murine β-defensin Defr1. All peptides showed broad-spectrum salt-sensitive antibacterial activity against Gram-positive and Gram-negative bacteria, but the antibacterial activities were found to vary between different peptides, in terms of both the spectrum of activity and the degree of salt-sensitivity. The data presented on the activity of DEFB4 is in broad agreement with previous studies that DEFB4 possesses high levels of salt-sensitive antimicrobial activity against the opportunistic and CF pathogen *P. aeruginosa*. The synthetic DEFB4 peptide used in this study was also shown to have weaker activity against another common pathogen *S. aureus*; however, activity against *E. coli* was strain specific. Defb2 demonstrated lower levels of antibacterial activity compared to DEFB4 against *S. aureus* and *P. aeruginosa* but the activities against *E. coli* were highly similar. That DEFB4 tended to be more efficient antimicrobial than Defb2 may be due to the fact that the pathogens analysed in this study mostly are recognised as human pathogens. It would be of interest therefore to investigate the activities of these two peptides against murine specific pathogens.

Furthermore, it would also be of interest to investigate if the function of different β-defensin peptides has become specialised to certain bacteria likely to invade the tissue in which that β-defensin is expressed. However, as DEFB4 is not the orthologue of Defb2 differences in the activities of the two peptides would be expected and therefore it would be interesting to compare of the activity of DEFB4 with the murine orthologue Defb4, and likewise for Defb2 and the human ortholgue if one is identified.

The antimicrobial activity of a second batch of synthetic Defb2 (Defb2^{2nd}) was also analysed as a control for further antibacterial experiments (see below). The two separate batches of Defb2 showed very similar levels of antibacterial activity against *E. coli* and *S. aureus*, but the activity against *P. aeruginosa* differed significantly. The first batch killed *P. aeruginosa* significantly less efficiently than the Defb2^{2nd}. Structural studies suggested that the different levels of activity may have been due to differently folded peptides.

The antibacterial activity of the novel murine β-defensin Defr1 was also analysed; this peptide lacks the first canonical cysteine that is present in all other β-defensins and this may have resulted in alteration of the antimicrobial activity. Defr1 showed high levels of antibacterial activity against *S. aureus* and *E. coli* in the absence of salt, however, the activity was markedly reduced by the presence salt. Interestingly, Defr1 demonstrated highly potent antimicrobial activity against *P. aeruginosa.* This activity was salt-insensitive at a peptide concentration of 50 μg/ml, but antimicrobial activity was inhibited by salt at a lower peptide concentration (5 μg/ml). This suggests that Defr1 is a highly potent anti-pseudomonal agent with levels of activity comparable to DEFB104 (Garcia *et al.*, 2001b). Of great interest was

the observation that Defr1 showed low levels of antibacterial activity the opportunistic pathogen *B. cepacia* whereas neither DEFB4 nor Defb2 activity. Indeed, this pathogen is resistant to the action of most antibacterial peptides and only one other defensin, DEFB103, (Garcia *et al.*, 2001a) has been shown to have antimicrobial activity against *B. cepacia*. Structural studies indicated that Defr1 exists in both monomeric and dimeric structural forms, whereas DEFB4 and Defb2 exist only in monomeric form. It is suggested that the potent antimicrobial activity may be associated due to the dimeric structure; although further investigation of this issue is required.

In view of the potent antimicrobial activity of Defr1, an interesting line of future work would be to investigate the relative activities of the monomeric and dimeric isoforms. It may be possible to isolate the different structural forms following their electrophoresis on a non-denaturing gel. However, it may be necessary to establish different conditions for peptide folding to generate the dimeric and monomeric forms separately. As discussed above, the Defr1 peptide lacks the first cysteine present in other β-defensins and this has resulted in an altered arrangement of disulfide bridges in the defensin molecule. Consequently, elucidation of the cysteine arrangement and the relative antibacterial activity of the different bridge-arrangements is also of great interest and may help explain the potent microbicidal activity of Defr1. Furthermore, investigation of the mechanism of antibacterial activity of Defr1, and indeed other β-defensins such as DEFB4 and Defb2, may also be served by investigation peptide-membrane interactions. Such studies have been performed for other antimicrobial peptides using lipid vesicles composed of known and varied lipid compositions.

Another fruitful area of research would be mutational studies comparing the antibacterial activity of the wildtype β-defensin peptides against various β-defensins with altered residues. This may be possible using β-defensins produced in bacteria using a His-tag system that has been previously used to produce recombinant DEFB103 peptides (Schibli *et al.*, 2002) (Harder *et al.*, 2001). The antibacterial activity and structure of the mutated peptides can be then be analysed and such studies may generate insights into the role of specific residues in the antibacterial activity. For example, the normally conserved cysteine could be introduced into Defr1, or conversely the first cysteine could be knocked out of Defb2 and the effects of these changes on antibacterial activity could then be assessed. Such studies may reveal if the unusual structure of Defr1 is connected with it potent antimicrobial activity. Another possible line of investigation would be to alter the cationicity of β-defensin peptides, because if the activity of β-defensins is driven by the overall charge of the peptide, then such alterations are likely to affect antibacterial activity.

Of particular interest are the observations that whereas Defr1 shows potent and salt-insensitive antibacterial activity against the Gram-negative *P. aeruginosa* and weak activity against the highly resistant *B. cenocepacia.* The human β-defensin DEFB103 shows potent and salt-insensitive antibacterial activity against the Gram-positive *S. aureus* and also shows antimicrobial activity against an environmental strain of *B. cepacia.* Clearly therefore, these two β-defensin peptides have some striking similarities in their activities and also some interesting differences. Given the innate resistance *B. cepacia complex species* to many conventional antibiotics and antimicrobial peptides it would be of particular interest to investigate the structural similarities

between these two peptides. Such investigations may aid in the elucidation of specific structural regions, residues and/or properties possessed by these peptides that may confer the observed potencies. Moreover, any potential functional regions can then be assessed using the mutational analysis described above or peptides could be further altered to generate more effective antimicrobial peptides that as potential therapeutic agents. These studies could be conducted using the *in vitro* assay used in this thesis and then progress to using *in vitro* primary cultures of airway epithelial cells followed by *in vivo* animal studies.

β-Defensins do not act in isolation *in vivo,* and therefore investigations of the interaction of β-defensins with other components of the immune system such as antimicrobial peptides (including other defensins) and cellular aspects of the immune response may further elucidate the role of these peptides under more physiological conditions. In concert with these studies, it would also be of interest to investigate the concentrations of β-defensins and salt at various epithelial surfaces; these issues are largely unresolved and clearly influence the effectiveness of these peptides as innate antibiotics. Moreover, mouse models in which the β-defensin loci have been knocked out would also prove enormously useful in further investigation of the function of these peptides as they would display the sum effects of the loss these peptides. However, due to the identification of several β-defensin loci the task would not be trivial. Although, if a complete knockout could be generated it may be possible to replace the murine β-defensins with human β-defensins as bacterial artificial chromosome (BAC) transgenes; this would provide a useful system for investigating the activity of human β-defensins in a physiological environment.

This thesis has also presented novel data on the chemoattractant activities of Defr1 and Defb2 for various immune-related cells. Defr1 and Defb2 were shown *in vitro* to induce migration of $CD4^+$ T-cells and immature dendritic cells, but not mature dendritic cells or neutrophils. This pattern of responses is in agreement with that seen with DEFB4 in previous studies (Yang *et al.*, 1999). Cell responses occurred at lower concentrations than the antibacterial activity (nanomolar as opposed to micromolar) and thus may reflect a more physiological concentration. However, further studies are required to investigate if these responses are chemotactic or chemokinetic. Experiments to address this can be performed using a checkerboard assay, although these are not ideal and so this issue may be better addressed using the chemotaxis chamber designed by Jeon *et al.* (2002) that establishes gradients of chemoattractant substances. Furthermore, previous studies have suggested that there is a dichotomy between the subtypes of $CD4^+$ T-cells attracted by α-defensins (naïve T-cells) and β-defensins (memory T-cells) and this is clearly an area for future investigation. Moreover, the populations of dendritic cells used in the studies presented here did not have a high level of purity, which may have adversely affected the observed levels of migration. Consequently, therefore, it may be necessary to find an alternative source of dendritic cells that will provide a population of acceptable purity, and this can be done by depleting contaminating cells from the bone marrow cultures by antibody-dependent complement-mediated depletion. The migratory activities of neutrophils, $CD4^+$ T-cells and dendritic cells were analysed in this thesis and there is clearly much scope to investigate the migration of other cell types, such cell types could include $CD8^+$ T-cells, B-cells, mast cell and monocytes/macrophages, and also to investigate which receptor is

involved in cell migration. Moreover, the effect of Defb2 and Deftr1 on other functions of immune cells (e.g. degranulation, cytokine production) could also be analysed.

The mutational studies described above can also be used for studies to investigate the chemoattractant activities of Defr1 and Defb2; for example, it is not clear what form or forms (*i.e.* monomeric or dimeric) of Defr1 acts as the attractant. For example, a conserved region of DEFB4 has been found shown to have homology with the chemokine MIP3α, and to mediate the chemotactic activities of DEFB4 and some of these properties are also conserved, at least in the nature of the residue, in the murine β-defensins analysed in this thesis. Therefore, it would be of great interest to introduce amino acid changes in the corresponding region of Defb2 and Defr1 to investigate if this region plays a role in the chemoattractant for example by mutating the conserved glutamate residue for a positively charged amino acid such as arginine.

It would also be of interest to use animal models for the analysis of cell migration by defensins. Such studies would clearly be of more relevance to the physiological function of β-defensins as they would incorporate 'indirect chemotaxis' induced by defensins (e.g. defensins may induce production of a chemokine such as IL-8, resulting in migration cell type not directly activated by defensins). It may be possible to deliver β-defensins by subcutaneous injection or intratracheal instillation and then to investigate the cell types that are recruited.

This thesis also described an attempt to establish a stably transfected cell culture-based system for the production of murine and human β-defensins. Mouse mammary C127 cell lines were established that stably expressed various human and mouse β-defensins. However, sufficient levels of β-defensins were not detected to make this system of use in future work in that it did not display increased antibacterial activity compared to the untransfected cell line. Moreover, expression of the defensins was not detectable by northern analysis, which may further suggest that the β-defensin were not produced at sufficient levels. It is quite possible that cell lines that expressed high levels of defensin suffered a growth disadvantage compared to non-expressing or low expressing lines, which may therefore have come to dominate the cell culture. Therefore, this work, if repeated may benefit from use of the repressor plasmid system. However, since the conception of this aspect of this thesis separate studies have suggested that sufficient quantities of functional and correctly folded β-defensins can be produced in bacteria. Such a system would ideal be given that it can produce sufficient quantities of peptide for analysis of antibacterial activity and also in light of the future work suggested above in that it can be used in conjunction with *in vitro* site-directed mutagenesis system. However, it may also be advantageous to use virally infected mammalian cell cultures to produce β-defensins *in situ,* such studies may generate tissue –specific N-terminal splice variants of the β-defensins. Moreover, viral-based system could be used to infect primary cultures of mammalian tracheal cells that could subsequently be used to analyse the antibacterial activity of β-defensins produced *in situ* on a functional epithelial surface.

Finally, this thesis has presented data on the expression patterns of five novel human β-defensins. All were found to be expressed at high levels in the testis and some were also present at much lower levels in other tissues. The sequence and frequency of potential polymorphisms in these genes needs to be confirmed by further sequencing. The RT-PCR analysis presented in this thesis shows that these novels genes are expressed, which may suggest that they are not pseudogenes. A clear future strand for this project is to analyse the antibacterial activity of these peptides as for previous β-defensins. Further characterisation of these genes is required in particular in DEFB109, which yielded two transcripts and DEFB107 lacks the canonical cysteine and in light of the striking function of Defr1, investigation of the activity of this peptide is of particular interest.

In summary, the studies presented in this thesis have demonstrated that β-defensins possess salt-sensitive antimicrobial activity against Gram-positive and Gram-negative bacteria. Moreover, they have shown that the loss of the normally conserved first canonical cysteine in Defr1 does not result in loss of activity. They have also demonstrated that Defr1 is a highly potent anti-pseudomonal agent and has antimicrobial activity against *B. cenocepacia* – an opportunistic pathogen that is normally resistant to antimicrobial peptides and causes severe problems ion cystic fibrosis patients. Furthermore, these studies have suggested that Defb2 and Defr1 are chemoattractants for $CD4^+$ T-cells and dendritic cells and may provide a link between the innate and adaptive immune system. Finally, this projected has confirmed the expression of several novel human β-defensins, which further suggest that the β-defensins are a large and only partially characterised subfamily of antimicrobial peptides.

8. References

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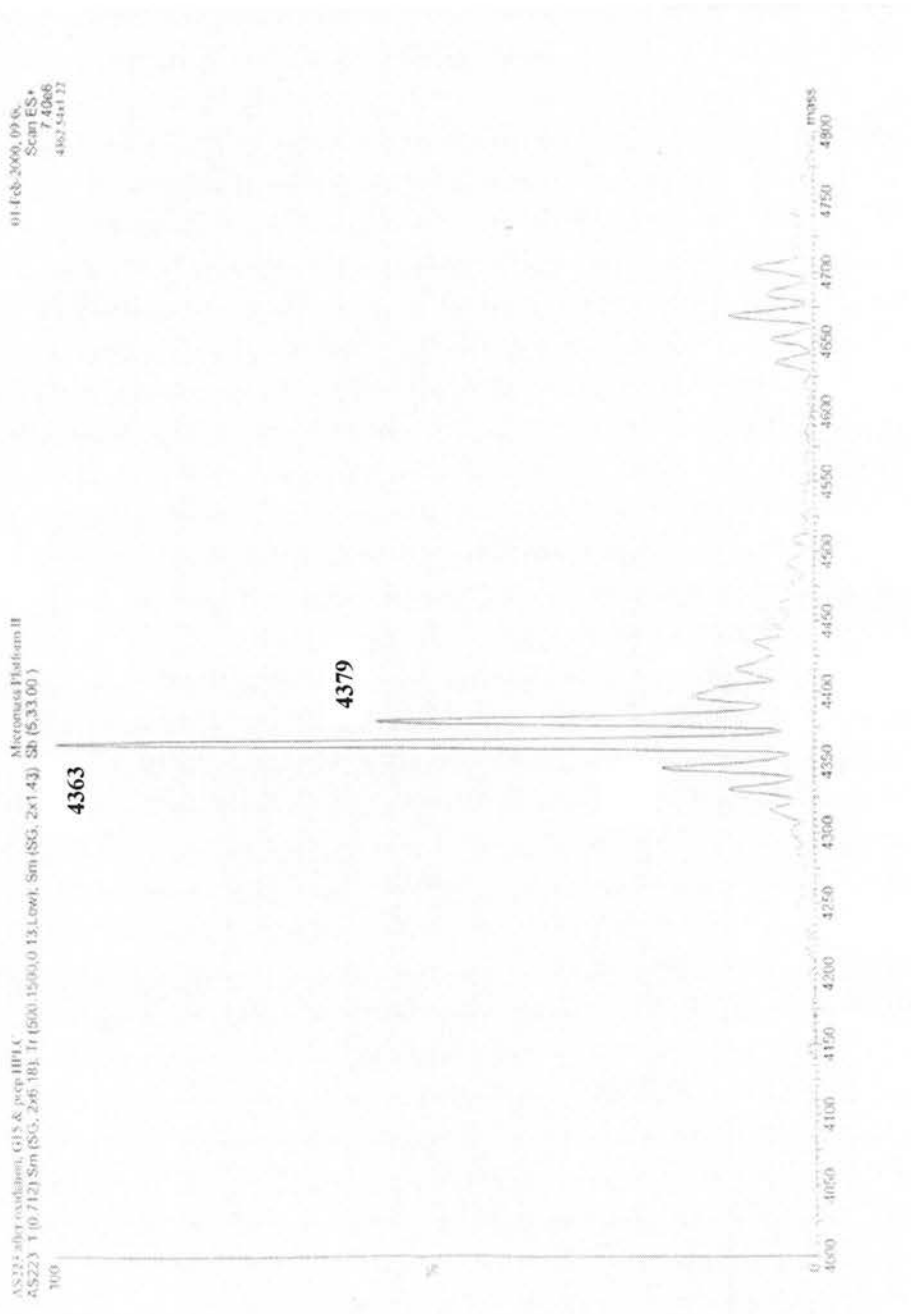
Appendix I

MALDI-TOF Mass Spectograms of Synthetic Defb2

First batch of Defb2



Defb2^{2nd}

AS328, folded, cv=50
AS328 1 (0.490) Tr (500,1600,1.00,Low); Sm (SG, 2x1.70); Sb (10,10.00)
31-May-2001, 17:10:25
Scan ES+
1.55e8
A: 4362.41±0.95
4363
4379
100
%
0
3900
3950
4000
4050
4100
4150
4200
4250
4300
4350
4400
4450
4500
4550
4600
4650
4700
4750
mass